Studies on plant based dietary supplements for control of *Aeromonas hydrophila* infections in rainbow trout (*Oncorhynchus mykiss* Walbaum)

Elham S. Awad

A thesis submitted for the degree of

Doctor of Philosophy

School of Life Sciences, Heriot Watt University, Edinburgh, UK

August 2010

Abstract

Rainbow trout (*Oncorhynchus mykiss Walbaum)* of average weight 18 g, were fed for two weeks with diet supplemented with 0.5 g (= 0.5%), 1 g (= 1%) and 2 g (= 2%) 100 g^{-1} of lupin (*Lupinus perennis)*, mango (*Mangifera indica*) and stinging nettle (*Urtica dioica*), and with normal diet as controls. Following challenge with *Aeromonas hydrophila,* there was a reduction in mortality compared with the controls. Furthermore, the fish, which were fed with dietary supplements, recorded enhanced haematological and immunological parameters including phagocytic, respiratory burst, lysozyme, bactericidal, complement, myloperoxidase and antiprotease activities, and total protein, compared to the controls. Feeding for 2 months with 1% and 2% of the dietary supplements led to an increase in growth performance, body composition, digestive enzyme (total proteases, amylase, lipase and pepsin), total protein and glucose compared to controls, whereas there was not any effect on kidney or liver function. The expression of IL-1ß, IL-8 and TGF- ß1 in head kidney of rainbow trout fed for 2 months was upregulated after feeding with 1% lupin and 1% stinging nettle. In contrast, there was not any significant effect following feeding with mango.

Dedication

To my lovely Mama, my Dad, and Professor Nadia Helmy

Acknowledgements

I would like to acknowledge my lord for his mercies upon me and all kind support to finish this work.

I would like to thanks my supervisor Professor Brian Austin for his help and useful advice during the research work as well as his encouragement.

I am grateful to Doctor Dawn Austin for her generous support, care, and encouragement throughout the study period. I will never forget her beautiful heart.

I would like to acknowledge Doctor Alastair Lyndon for his kind help and useful advice especially in the work concerning digestive enzyme.

Many thanks for Dr. Karim Rodor, for his useful advices and his continuing help during the study. I really appreciate all that what he did for me.

I would like to thanks Doctor Wilf Mitchell for his expertise and guidance with RNA extraction and the molecular part of the study. Deep acknowledgement and gratitude go to Dr Colin Moore, for his guidance with the statistical analyses. Also I would like to thanks all staff members and colleagues; Dr. Peter, Dr. Amany, Margaret, Paty, Lionel, Majde, Mohamed, Salem, Wael, Tarek, John, Sharif, Bayani, Gina, Alison, Sean, Rob, Vicky and Colin.

Thanks is not enough for my best friend Samia for her support and encouragement. Also I would like to thanks my friends, Eman, Esraa, Heba, Rania, Samah.

I am thankful for the support which I receive it from my family, Mam, Dad, Amani, Ashhab, Asmaa, and Eslam.

Finally I would like to thank the Egyptian Government for their financial support

ACADEMIC REGISTRY
Research Thesis Submission

Name*:*	Elham S. Awad		
School/PGI:	Life Sciences		
Version: *(i.e. First, Resubmission, Final)*	First	Degree Sought (Award **and** Subject area)	Ph.D.

Declaration

In accordance with the appropriate regulations I hereby submit my thesis and I declare that:

1) The thesis embodies the results of my own work and has been composed by myself
2) Where appropriate, I have made acknowledgement of the work of others and have made reference to work carried out in collaboration with other persons
3) The thesis is the correct version of the thesis for submission and is the same version as any electronic versions submitted*.
4) my thesis for the award referred to, deposited in the Heriot-Watt University Library, should be made available for loan or photocopying and be available via the Institutional Repository, subject to such conditions as the Librarian may require
5) I understand that as a student of the University I am required to abide by the Regulations of the University and to conform to its discipline.

** Please note that it is the responsibility of the candidate to ensure that the correct version of the thesis is submitted.*

Signatureof Candidate*:*		Date:	

Submission

Submitted By *(name in capitals):*	ELHAM S. AWAD
Signature of Individual Submitting:	
Date Submitted:	

For Completion in Academic Registry

Received in the Academic Registry by *(name in capitals):*			
Method of Submission *(Handed in to Academic Registry; posted through internal/external mail):*			
*E-thesis Submitted (**mandatory from January 2010)***			
Signature:		Date:	

Declaration

I, Elham Awad, hereby declare that I am the author of this thesis. All the work described in this thesis is my own except where started in the text. The work presented here has not been accepted in any previous application for a higher degree. All the sources of information have been consulted by myself, and are acknowledged by means of references.

Elham Awad

Contents

Results

List of Tables

List of Figures

List of abbreviation and symbols

~	Approximately
<	Less than
>	More than
≥	More than or equal to
µl	Microlitre
ACP	Alternative complement pathway
Ag-Ig	Antibody-antigen complex
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
B- cells	Bone cells
BC	Before Christ
bp	Base pair
BSA	Bovine serum albumin
Ca	Calcium
CCP	Classical complement pathway
cDNA	Complementary Deoxyribonucleic acid
CF	Condition factors
CRP	C- reactive protein
C_T	Threshold cycle
dH_2O	Distilled water
DIR	Daily intake rate
dl	Decilitre
DMSO	Dimethyl sulphoxide
dNTP	Deoxyribonucleotide
E	Efficiency
ECP	Extracellular products
ERM	Enteric redmouth disease
et al	'et alia' and others
ETE	*Ecteinascidia turhinuru* extract
FAO	Food and Agriculture Organization of the United Nations
FCA	Freund's complete adjuvant
FCR	Feed conversion ratio
FCS	Foetal calf serum
FDA	Food and Drug Administration
Fe	Iron
FK-565	Heptanoyl-y-D-glutamyl-(L)-meso-diaminopimelyl-(D)-alanine
g	Gram
GOT	Glutamic pyruvic transaminase
GPT	Glutamic pyruvic transaminase
h	Hour
H_2SO_4	Sulphuric acid
Hb	Haemoglobin
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
Hct	Haematocrit
HDe	*Haliotis discus* extract
HM	Medicinal herb
i.e.	That is
IFN	Interferons

Ig	Immunoglobulin
IL-1	Interleukin -1
IL-8	Interleukin- 8
ISK	Fish extract
K	Potassium
KCl	Potassium chloride
Kg	Kilogram
KOH	Potassium hydroxide
Kps	Kilobasepair
L	Litre
LCP	Lectin complement pathway
LPS	Lipopolysaccharide
M	Molar
MDP	Muramyl dipeptide
Mg	Magnesium
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minute
ml	Millilitre
mM	Milimole
MOPS	3-(N-morpholino)propanesulfonic acid
MPO	Myeloperoxidase
Na	Sodium
NaOH	Sodium hydroxide
NBT	Nitroblue tetrazolium
NK	Natural killer
nm	Nanometre
ºC	Degree Celsius
OD	Optical density
OH	Hydroxide
PA	Phagocytic activity
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ppm	Part per million
RaRBC	Rabbit red blood cells
RBC	Red blood cells
RFU	Relative fluorescence units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Rotation/revolutions per minute
RPS	Relative percent survival
rRNA	Ribosomal ribonucleic acid
sec	Second
SGR	Specific growth rate
spp.	Species
T- cells	Thymus cells
TAE	Tris acetic acid
Tc	Cytotoxic T-cells
TCA	Trichloro acetic acid
TCM	Traditional Chinese medicine
TGF	Transforming growth factors

Th	Helper T-cells
Tk.	Tone kilogram
T_m	Melting temperature
TMB	3,3´,5,5´- tetramethylbenzidine hydrochloride
TNF	Tumor necrosis factor
TSA	Tryptone soya agar
TSB	Tryptone soya broth
U	Unit
UK	United kingdom
USA	United States of America
V	Volt
v/v	Volume to volume ratio
w/v	Weight to volume ratio
WBC	White blood cell
Zn	Zinc
µg	Microgram
µm	Micrometre

Publications

Awad, E. and Austin B. 2009. Use of lupin (*Lupinus perennis*), mango (*Mangifera indica*) and stinging nettle (*Urtica dioica*) as feed additives to prevent *Aeromonas hydrophila* infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum). J. Fish Dis. 33, 413 – 420

Awad, E. and Austin B. 2010. Effect of dietary supplements on digestive enzymes and growth performance of rainbow trout (*Oncorhynchus mykiss*, Walbaum). J. Fish Bio. (In press).

Awad, E., Mitchell, W.J. and Austin B. Effect of dietary supplements on cytokine gene expression in rainbow trout (*Oncorhynchus mykiss*, Walbaum). J. Fish Dis. (To be submitted).

Abd El-Baky, S.M.M., Helmy, N., El Abbas, A., Abdel Mohsen, A. and Awaad, E.S. 2006. Prevalence of borrelial infections in tick vectors and vertebrate hosts in Esmaieliya governorate, Egypt. J.Agric .Sci. Mansoura Univ. 31, 2387-2403.

El Abbas, A., Abd El-Baky, S.M.M., Helmy, N. Abdel Mohsen, A. and Awad, E.S. 2006. Seasonal abundance of hard ticks (Ixodidae) in two localities (Giza and Esmaieliya governorates) of Egypt. Sci. J. Fac. Sci. Minoufia Univ.,Vol.XX:65-87.

Helmy, N., El Abbas, A., Abd El-Baky, S.M.M., Abdel Mohsen, A. and Awad, E.S. 2006. Prevalence of borrelial infections in tick vectors and vertebrate hosts in Giza governorate, Egypt . Sci. J. Fac. Sci. Minoufia Univ. Vol. XX:101-131.

Introduction

1.1 Aquaculture

1.1.1 Definition

The word ‘aquaculture’ derives from the Latin ‘aqua’ (= water) and ‘culture’ (= cultivation, especially with a view to improvement). The term ‘aquaculture’ covers all activities aimed at producing, processing and marketing aquatic plants and animals from fresh, brackish and salt waters. In narrow terms, aquaculture includes the use and manipulation of natural and artificial bodies of water to produce species required by man, and thus is concerned with all activities concerned with breeding and culture of aquatic organisms. Aquaculture has been defined by the Japanese Resource Council, Science and Technology Agency as: “Aquaculture is an industrial process of raising aquatic organisms up to final commercial production within properly partitioned aquatic areas, controlling the environmental factors and administering the life history of the organism positively and it has to be considered as an independent industry from the fisheries hitherto.” Aquaculture is the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants with some sort of intervention in the rearing process to enhance production, such as regular stocking, feeding, and protection from predators, parasites and pathogens. Farming also implies individual or corporate ownership of the stock being cultivated (as defined by the Food and Agriculture Organization of the United Nations; FAO).

1.1.2 History of aquaculture

Most publications on aquaculture refer to the long history of fish culture in China, ancient Egypt and central Europe. The earliest record of fish farming was in 2500 BC in China. This early farming activity involved capturing fish, mainly carp, after river floods and holding them in artificial lakes and ponds. Hieroglyphics illustrate that the Egyptians of the Middle Kingdom (2052-1786 B.C.) developed ornamental fish ponds and attempted intensive fish culturing. Also, research indicates that the Roman's were quite adept in raising fish in ponds, and also cultivated oysters. Similarly, the Hawaiian people practiced aquaculture by constructing fish ponds; an example from ancient Hawaii is a pond at Alekoko dating back at least 1000 years (Pillay & Kutty, 2005).

The propagation of trout, which has a fairly long history, originated in France, and the monk Don Pinchot, who lived in the 14th century, is credited with the discovery of the method of artificial impregnation of trout eggs (Davis, 1956). Being a sport fish and more widely accepted for its culinary properties, trout culture spread to almost all continents in the course of time. Commercial trout culture in fresh water on a fairly large scale was developed in countries such as France, Denmark and Japan, and later in Italy and Norway. The British introduced trout in their colonies in Asia and Africa. Culture in North America was centred on the propagation of salmon and trout, and to a lesser extent on black bass. Starting in the 18th century, trout hatcheries were established in government stations mainly for release of fry into open water, but in the course of time the private sector started commercial production for culinary purposes.

A Danish trout farmer in the early 1900's developed a pioneering farm design where fresh water flowed through each fish pond to improve fish yield and reduce infectious disease. This breakthrough signified the beginning of the commercial trout-for-table farming industry to reach 360 trout farms (in Denmark) producing around 16,000 tonnes per annum (http://www.westlowmere.co.uk/photo's.html).

In England and Wales, there are approximately 500 registered fish and shellfish farms. Of these, 193 are coarse fish farms, 197 trout and other fin fish farms and 128 shellfish sites. The main finfish species farmed is rainbow trout (annual production = 7,294 tonnes) as compared with other species, such as brown trout (441 tonnes), carp (175 tonnes) Atlantic salmon (63 tonnes), turbot (63.5 tonnes), barramundi (45 tonnes), tilapia (33 tonnes), for a total fish farm production in England and Wales of 8,127 tonnes. In 2006, shellfish farm production was around 15,449 tonnes, the main species were mussels (14,553 tonnes) and oysters (880 tonnes) (http://www.defra.gov.uk/foodfarm/fisheries/documents/aquaculture-report0904.pdf).

Scotland is responsible for 80% of the UK aquaculture production. Scottish production of Atlantic salmon in 2007 was ~129,930 tonnes, followed by rainbow trout (7,414 tonnes), cod (1,111 tonnes), brown trout/sea trout (124 tonnes), halibut (147 tonnes) and Arctic charr (6.5 tonnes). In 2008, rainbow trout production increased by 256 tonnes according to the latest Scottish Fish Farms Production Survey

(http://www.thefishsite.com/fishnews/11496/scotland-increase-rainbow-trout-production).

1.2 Rainbow trout (*Oncorhynchus mykiss*)

Rainbow trout, *Oncorhynchus mykiss,* is a species of the family *Salmonidae. Oncorhynchus* refers to "hooked nose", and *mykiss* refers to a Siberian word for the species. It was named by Johann Julius Walbaum in 1792, based on type specimens from Kamchatka in Russia. Previously, the fish was named *Salmo gairdneri,* but in 1992 the genus name was changed from *Salmo* to *Oncorhynchus* (http://aqua-culture.blogspot.com/2007/01/rainbow-trout-salmo-gairdneri.html).

Many countries report rainbow trout farming production. Some of them have relatively insignificant output in comparison to the production from the larger systems that are located in the primary producing areas in Europe, North America, Chile, Japan and Australia (Fig. 1.1). Rainbow trout was introduced to Britain from western North America in 1885 (MacCrimmon, 1971). Fast-growing and tolerant of crowding in captivity, they are now widely used around the world in farming for culinary use and for the restocking of fisheries. Approximately 16,000 tonnes of rainbow trout are produced in Britain each year, with ~75% of this farmed by table producers. Most of this production is in freshwater tanks, ponds, cages and raceways, with a small quantity farmed in sea cages. Trout is farmed widely in the UK (Fig. 1.2), but particularly in central and southern Scotland (Fig. 1.3), southern England and North Yorkshire.

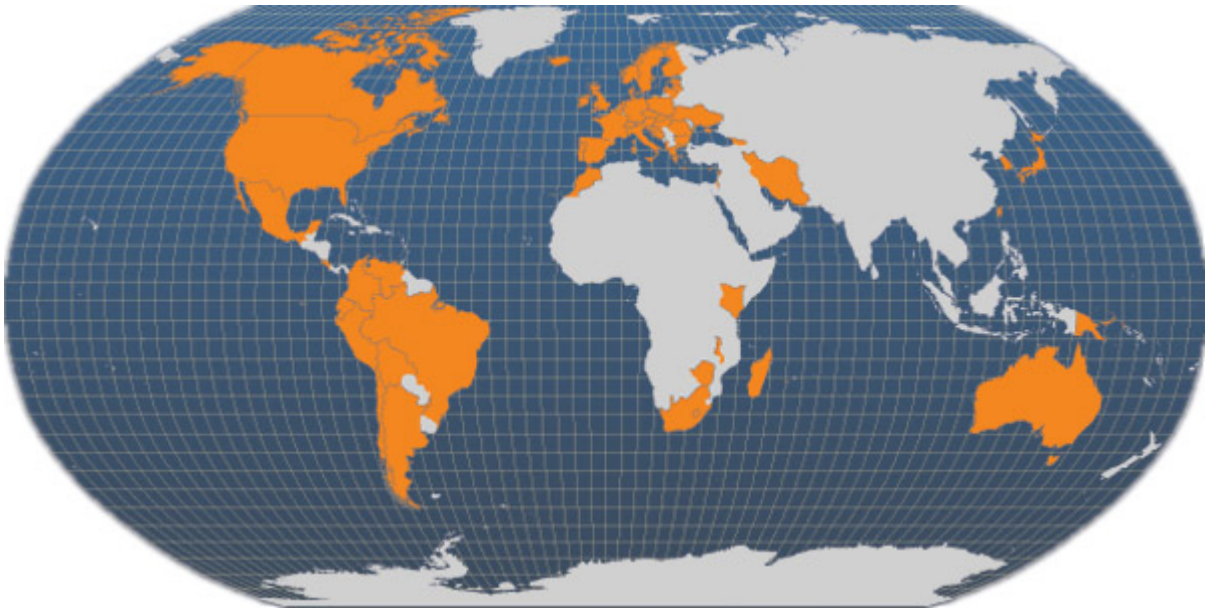


Fig. 1.1 Main producers of rainbow trout (Source: FAO Fishery Statistics, 2006)

Fig. 1.2 Map of trout farms in Great British (Source: British Trout Association).

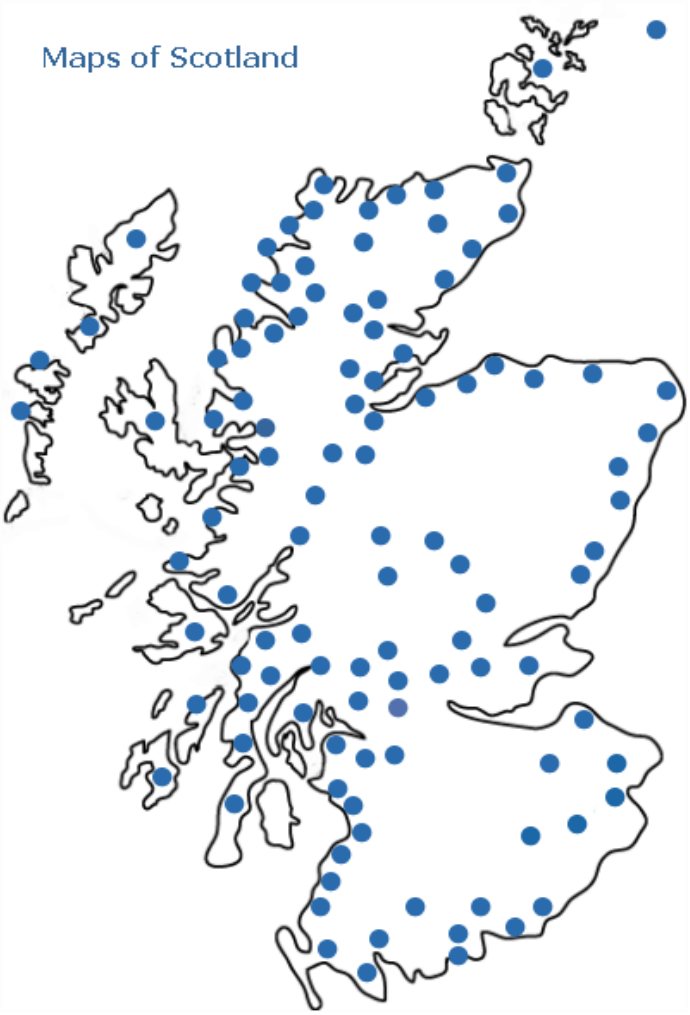


Fig. 1.3 The map shows the main trout, salmon and sea trout fishing areas in Scotland (with acknowledgements to www.trout-salmon-fishing.com/maps-scotland.htm)

1.3 Diseases in aquaculture

Marine life is declining from overfishing, pollution, habitat destruction and global warming. For those reasons, aquaculture is rapidly increasing its annual global harvest and seems to offer hope for increased food production, which has led to the widespread use of intensive fish culture practices (Lewis & Galtin, 2004**).** A survey done in Bangladesh during 2003 showed average economic losses of Tk.20,615/ha/year (US$ 344) to farmers from fish diseases, which was equivalent to ~15% of the actual production (Faruk *et al.*, 2004). Overall, fish health problems in farmed fish, especially disease problems, are directly linked to stress on the fish from environmental factors (= stressors) disrupting physiology (Table 1.1). Thus, there are direct relationships between stress, fish health and disease (Schmittou *et al.*, 2004) (Fig. 1.4). Moreover, stress is considered as the major factor which affects fish health, and may lead to infectious disease. Stressors can be acute or chronic, and their impacts on fish are additive and

cumulative, at least for a short period (Schmittou *et al*., 2004**).** Stressors may be chemical, biological, physical or procedural (Fig. 1.5). Higher densities of fish are more likely to have problems with pathogens, and may promote genetic selection of mutant pathogens that are more virulent (Owens, 2003). However, fish vary in their resistance to infectious disease because of the genetic composition and/or as a result of previous exposure to pathogens and thus the development of protective immunity. Biological stressors, especially viruses and bacteria, are recognized as the aetiological agents of infectious diseases in a wide variety of fish species (Austin, 2005).

Table 1.1 Biomass (tonnes) and economic losses (millions of Yen) in Japanese aquaculture attributed to various biological agents in 1993 (after Sano, 1998)

Category of causative agent	**Biomass loss**		**Economic loss**	
	Tonnes	%	Yen	%
Bacteria	11197	74.3	12417	69.4
Viral	1197	2.7	3572	20.0
Concurrent	702	4.7	342	1.9
Parasitic	579	3.8	932	5.2
Fungus	184	1.2	192	1.1
Others	495	3.3	443	2.5
Total	15079	100	17898	100

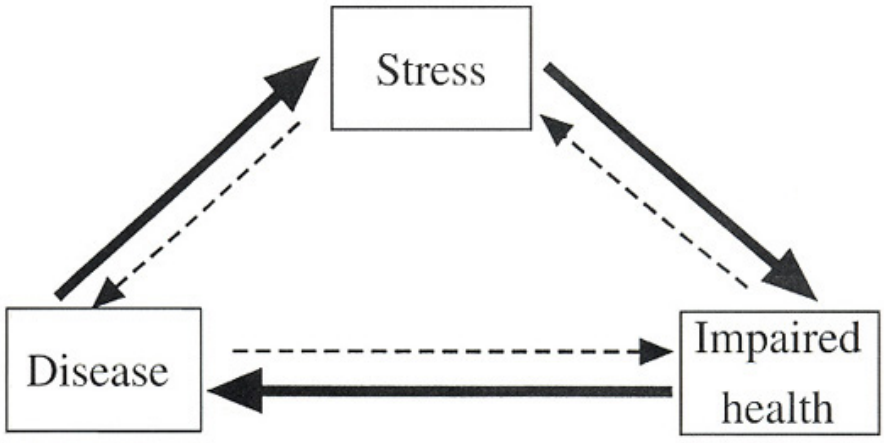


Fig. 1.4 Chart illustrating the direct interrelationships between fish stress, health and disease (after Schmittou *et al*., 2004**)**

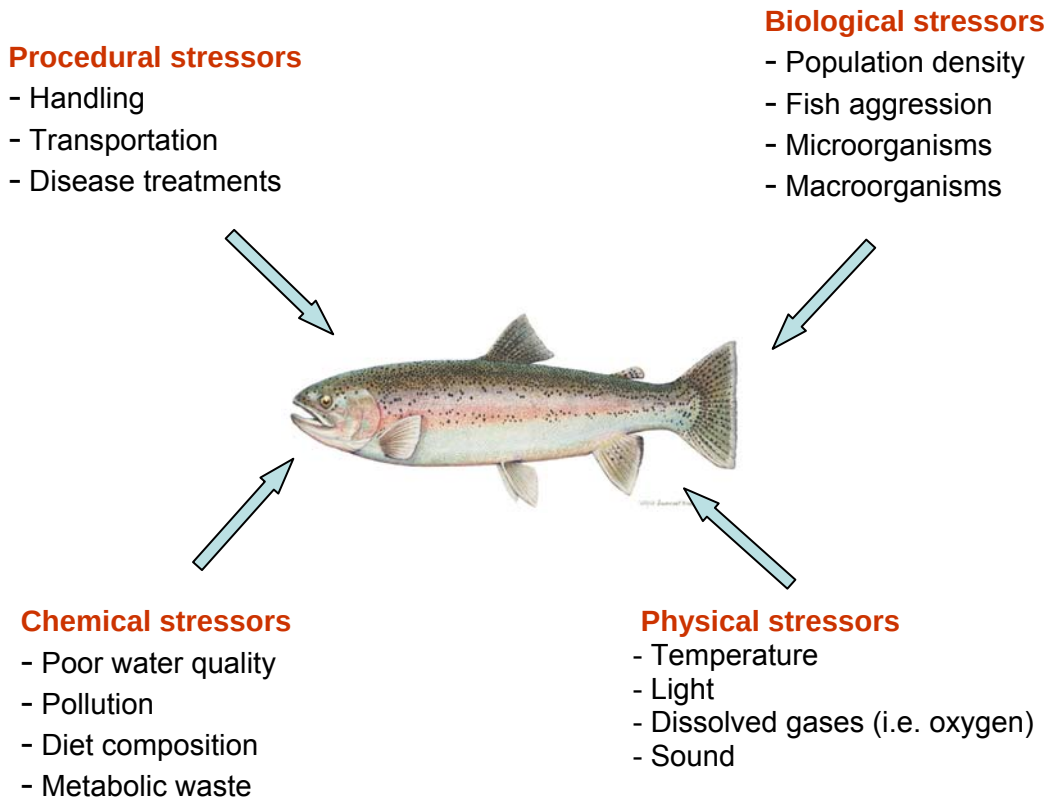


Fig. 1.5 Illustration of the different types of stressors affecting fish.

1.4 Bacterial diseases in aquaculture

The severity of diseases is influenced by a number of interrelated factors, including bacterial virulence, the kind and degree of stress pressed on fish, the physiological condition of the host, and the degree of genetic resistance inherent within specific populations of fish (Cipriano *et al*., 2001**).** A wide variety of bacterial pathogens have been associated with fish disease (Austin & Austin, 2007). For example, *Renibacterium salmoninarum* (bacterial kidney disease; Dee disease, renibacterial kidney disease), *Edwardsiella* spp. (edwardsiellosis). *Vibrio* spp. (vibriosis), *Aeromonas salmonicida* (furunculosis; ulcer disease), *A. hydrophila* (haemorrhagic septicaemia; fin/tail rot), *Yersinia ruckeri* (enteric redmouth disease, ERM) and *Flexibacter* spp. (gill disease, black patch necrosis) have been reported to cause disease leading to high mortalities.

Bacterial infections caused by members of the genus *Aeromonas*, which have a relatively high antibiotic resistance, are among the most common diseases of fish especially in pond systems with recirculation (Saavedra *et al*., 2004). For example, *A. salmonicida*, the causative agent of furunculosis, is a devastating disease of salmonids

(Austin & Austin, 2007). *A. hydrophila* is widely distributed in aquatic environments (Palumbo *et al*., 1992; Yu *et al.*, 2004), and causes bacterial haemorrhagic septicaemia and epizootic ulcerative syndrome in fresh water (Shao *et al*., 2004) and marine species (Lilley *et al*., 1997). Vibriosis is one of the most infamous fish diseases caused by *Vibrio anguillarum*, and affects marine and freshwater fish species (Kusuda & Salati, 1993). *Yersinia ruckeri* is the causal agent of enteric redmouth disease (ERM), which is characterized by reddening of the mouth and throat caused by subcutaneous haemorrhaging and petechial haemorrhages on the internal organs and the intestine becomes filled with an opaque, yellow, mucoid or watery material; the intestinal structure becomes flaccid (Austin & Austin, 2007).

1.5 *Aeromonas hydrophila*

1.5.1 Background

A. hydrophila comprises Gram negative motile, straight rods of 0.3-1.0 x 1.0- 3.5 μm in size. The organism belongs to the family Aeromonadaceae. The first report of isolation of *A. hydrophila* was by Sanarelli (1891), and it has developed into the most common organism in freshwater and occasionally marine fish (Larsen & Jensen, 1977; Cipriano *et al*., 2001; Pianetti *et al*., 2005). Also the organism has been isolated from diseased frogs (Gibbs, 1963), alligators (Shotts *et al*., 1972), snails (Mead, 1969), and freshwater prawns (De Figueredo & Plumb, 1977). Elevation in water temperature (Groberg *et al*., 1978), the presence of pollutants and intensive production systems are responsible for increasing the susceptibility to infection (Hanson & Grizzle, 1985). Although it forms part of the intestinal flora of healthy fish (Newman, 1983; Holmes *et al*., 1996), under stress conditions the organism becomes pathogenic (Cipriano *et al*., 2001**).** In Southeast Asia, fish kills due to *A. hydrophila* contribute a substantial economic loss to the fish farming industry (Llobrera & Gacutan, 1987; Thampuran *et al.,* 1995).

A. hydrophila can cause invasive fish disease (Austin & Austin, 2007) and has been isolated from lesions and tissues in diseased rainbow trout (Kapetanović & Teskeredžić, 2004). Isolation has been achieved using kidney swabs with non-selective media, such as nutrient agar or tryptone soya agar (TSA), or selective media, namely Rimler- Shotts medium (Shotts & Rimler, 1973) with incubation for 24-48 h at 20-25ºC. Both phenotypic and serologic methods are used in diagnosis (Austin & Austin, 2007). Glucose fermentation is regarded as a critical reaction for use in identification (Cipriano

et al., 2001). Moreover, many researchers and diagnosticians use the API 20E rapid identification system for routine identification (Overman *et al.,* 1985; Popovic *et al*., 2007).

1.5.2 Epizootiology

A. hydrophila is ubiquitous in fresh water (Allen *et al*., 1983) and fish eggs (Hansen & Olafsen, 1989). The organism can be found both in the water column and in the upper layer, i.e. top centimetre, of sediment (Hazen, 1979). Some isolates have demonstrated chemotactic responses to the mucus of freshwater fish (Hazen *et al*., 1982), and may initiate diseases in coldwater species (Cipriano *et al*., 2001). In the USA, most epizootics that occur among warm water fish are generally reported in spring and early summer (Meyer, 1970). In this connection, it is relevant to note that Osborne *et al.* (1989) found a high density of motile *Aeromonas* within the environment during mid summer when the water temperature was highest. Heavy mortalities were also correlated with aphanomycete-associated haemorrhagic septicaemia in rice field fish in South and South-East Asia (Roberts *et al.,* 1986).

1.5.3 Pathogenicity

The pathogenicity of *A. hydrophila* has been the focus of many investigations (e.g. De Figueredo & Plumb, 1977; Lallier *et al.,* 1981; Paniagua *et al.,* 1990; Wong *et al*., 1998; Richards & Parveen, 2005). The pathology attributed to members of the motile aeromonad complex include dermal ulceration, tail and/or fin rot, exophthalmia, erythrodermatitis, haemorrhagic septiceamia, red sore disease, red rot disease and scale protrusion disease (Cipriano *et al*., 2001; Austin & Austin, 2007) (Fig. 1.6)**.** An acute mortality was recorded in Nile tilapia infected with *A. hydrophila* in which the most apparent clinical signs included opaqueness in one or both eyes, accompanied by exophthalmia and eventual bursting of the orbit (Yambot & Inglis, 1994). Liver and kidney are commonly the target organs during acute septicaemia. Thus, Huizinga *et al.* (1979) reported the presence of pale liver and a greenish coloration in the kidney, which became swollen and friable after attack by *A. hydrophila*. Affected fish may show exophthalmia, reddening of the skin, an accumulation of fluid in the scale pockets, and distended abdomen as a result of oedema (Faktorovich, 1969). Chronic infections led to ulceration, in which dermal lesions with focal haemorrhages and inflammation were apparent (Cipriano *et al*., 2001).

In humans, five species of *Aeromonas* have been associated with disease, and the majority, i.e. > 85%, are attributed to *A. hydrophila, A. caviae* and *A. veronii*. The organisms may cause bacterial gastroenteritis, sepsis and bacteraemia in infants with multiple medical problems and in immunocompromised hosts, especially those with malignant or hepatobiliary diseases (Kasper & Barlam, 2005). Tulsidas *et al*. (2008) reported portal pyaemia secondary to *A. hydrophila* occurring in a 71-year-old Chinese man with fever, rigours and abdominal bloating. Clark & Chenoweth (2003) reviewed 126 patients from whom *Aeromonas* spp. were isolated over a seven-year period. They identified 17 episodes of involvement of the hepatobiliary system in 15 patients.

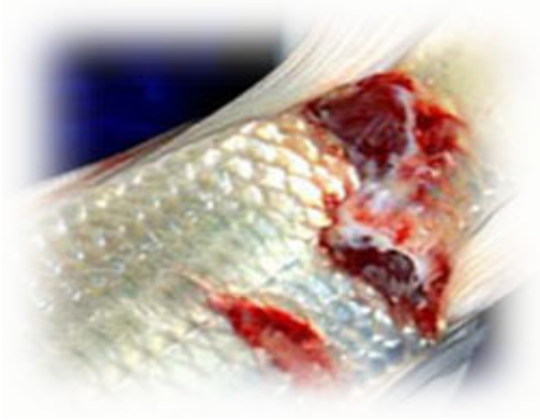
Fin /Tail rot

Skin ulcer

Distended abdomen

Exophthalmia

Scale protrusion



Haemorrhagic septicaemia

Fig. 1.6 Common symptoms of *Aeromonas hydrophila* infected fish.

1.5.4 Disease control - chemotherapy

Chemotherapy for *A. hydrophila* infections depends to a considerable extent on national regulations, with the approaches corresponding closely for that of *A. salmonicida* (Inglis *et al.,* 1993) (Table 1.2). A wide range of antibiotics has been found to have decreasing effectiveness because of resistance, and include ampicillin, chloramphenicol, erythromycin, nitofurantoin, novobiocin, streptomycin, sulphonamides and tetracycline

(Aoki, 1988; De Paola, 1988). Terramycin®, a brand of oxytetracycline, was approved by the Food and Drug Administration (FDA), USA to use in the production of salmonids, channel catfish and lobsters. Interestingly, Dopazo *et al.* (1988) found antibiotic-producing marine bacteria which showed inhibition to *A. hydrophila*. Moreover, orally administered piromidic acid was effective against experimental infections with *A. hydrophila* in goldfish and eels with the efficacy equal to or higher than that of chloramphenicol, and higher than that of tetracycline (Katae *et al.,* 1979). Although, furanace was extremely effective against *A. hydrophila* when catfish were immersed for 5 to 10 minutes in water containing 1-2 mg/l furanace, or by maintaining fish for 1 week in water containing 0.1 mg/l drug, there was an overriding problem of toxicity if used improperly (Mitchell & Plumb, 1980).

A major problem with the antibiotic treatment of *A. hydrophila* infections is the emergence of antibiotic/drug resistance bacterial strains which reduce or eliminate the effectiveness of the compound(s) (Aoki & Egusa, 1971; Mitchell & Plumb, 1980; Wooley *et al.*, 2004). Transferable R-plasmids are undoubtedly responsible for multiple drug resistance that has been described for aeromonad infections (Chang & Bolton, 1987; Ansary *et al*., 1992; Aruna & Chandran, 1996). Problems arise with accumulation of these inhibitory substances in the environment, and this has led to the emergence of resistant strains (Aruna & Chandran, 1996). Interestingly, exposure to one antibiotic may confer resistance to another compound. For example, *A. hydrophila* exposed to oxolinic acid developed cross-resistance towards flumequine and oxytetracycline (Hansan *et al*., 1992). Immunosuppression is another negative effect following use of antimicrobial compounds even at normal therapeutic concentrations (Lunden & Bylund, 2000). Nevertheless, the sensitivity of *A. hydrophila* to various types of antibiotics has been documented (Harikrishnan & Balasundaram, 2005) (Table 1.3).

Table 1.2 Treatments used to control *A. hydrophila* in fish

Treatment	Host species	Reference
Oxytetracycline and Tetracycline	Catfish (*Ictalurus punctatus*)	De Paola *et al*., 1988
Furanace	Catfish	Mitchell & Plumb, 1980
Piromidic acid	Goldfish and eels	Katae *et al*., 1979

Table 1.3 Sensitivity of *A. hydrophila* to antibiotics (adapted from Harikrishnan & Balasundaram, 2005**)**

Antibiotics	Reference	Sensitivity
Chloramphenicol	Ling-Yang & Chiu-Yung, 1987	S
Trimethoprim-sulphamethoxazole	Hirvelac-Koshi *et al.,* 1994	S
Nitrofurantoin		S
Oxolinic acid	Leano *et al.,* 1999	
	Hirvelac-Koshi *et al*., 1994	S
Ampicillin	Pasquale *et al*.,1994	R
Flumequine, Gentamycin, Nitrofurantoin, Trimethoprim,		
Oxytetracycline	Hettiarachchi & Cheong, 1994	R
Tetracycline	Nielsen *et al.,* 1994	R
	Pasquale *et al*., 1994	
Trimethoprim-sulphamethoxazole	Hettiarachchi & Cheong, 1994	R
	Pasquale *et al.,* 1994	
Streptomycin	Hettiarachchi & Cheong, 1994	R
	Baticados *et al*., 1990	
Penicillin	Hettiarachchi & Cheong, 1994	R
	Nielsen *et al.,* 1994	
	Baticados *et al.,* 1990	

R = resistance; S = sensitivity

1.6 Prophylactic methods to control disease

For the last 20 years, chemotherapy has been a dominant tool used in the management of diseases in aquaculture (Stoskopf, 1993). Although there has been the emergence of multiple drug resistant strains of pathogens, the antimicrobial compounds used in aquaculture have residual effects and undergo biomagnification in the aquatic environment particularly when used over an extended period of time (Mitchell & Plumb, 1980). For this reason, recently there has been a tendency to develop other approaches, including vaccines, probiotics and immunostimulants, for the control of fish diseases.

1.7 Vaccines

From the 1970's, interest in fish vaccines garnered interest, leading to the first commercial products for ERM and vibriosis (Harikrishnan & Balasundaram, 2005). The reasons for interest in developing vaccines were varied and included concern about the high cost of chemotherapy, the short-term nature of the protection obtained with antibiotics, the increasing appearance of antibiotic-resistant fish pathogens, and, to some extent, concerns about the environmental impacts of antibiotic use (Evelyn, 1997). Since, the first publication about the possibility of a vaccine for the control of furunculosis by Duff (1942), many investigations have sought to enhance immunity and control fish disease. The methods of vaccine preparation have varying effects, for example vaccination with crude lipopolysaccharide (LPS) induced better protection against *A. hydrophila* infection in the common carp, *Cyprinus carpio,* than a formalin killed vaccine (Baba *et al*., 1988). Success resulted from the study involving Indian major carps, *Catla catla, Labeo rohita*, and *Cirrhinus mrigala,* which were immunized intraperitoneally against *A. hydrophila* in field conditions using polyvalent antigen preparations from whole cell and extracellular products (ECP). The approach achieved high relative percent survival (RPS), i.e. 80–90% (Chandran *et al*., 2002). Certainly, the effectiveness of a vaccine will reflect the method of administration, which may be by oral, injection or immersion routes (Le Breton, 2009) (Table 1.4). Schachte (1978) recorded the effectiveness of immunization of channel catfish with a combined *A. hydrophila* and *Flexibacter columnaris* heat inactivated whole cell vaccine when administrated by injection or immersion but not by feeding. Anbarasu *et al.* (1998) found that formalin inactivated vaccines were superior to heat killed preparations, especially when the products were injected with adjuvants. Furthermore, Atlantic salmon immunized by intramuscular injection of extracellular proteases from *A. hydrophila* were protected from challenge with the homologous and some heterologous isolates of *A. hydrophila* (Shieh, 1987).

Consistently, oral vaccines have yielded low protection against diseases, and this is may be the reason for the general lack of effective commercial oral products for fish (Agius *et al*., 1983). The use of immunostimulants (= adjuvants) in vaccine vaccinations has been successful in leading to superior protection through heightened antibody response and elevation of nonspecific components of the immune system (Anderson, 1992; Raa, 1996; Sakai, 1999).

One of the major constraints in vaccination is that new diseases and pathogens emerge from time to time, and it is impossible to rapidly develop proactive prophylactic strategies using vaccines (Harikrishnan & Balasundaram, 2005). The wide range of antigens associated with motile *Aeromonas* markedly limits the development of vaccines against this group of bacteria (Newman, 1983; Stevenson, 1998). Certainly, one negative aspect of using vaccines is a decrease in growth performance (Lillehaug, 1991; Poppe & Breck, 1997) and induction of gross pathological changes, which detract from the aesthetic appearance of the fish (Midtlyng *et al*., 1996; Whittington *et al*., 1994).

Table 1.4 Vaccination methods: advantages and disadvantages (after Le Breton, 2009)

Vaccination method	Advantages	Disadvantages
Immersion vaccination: (1) Dip immersion (2) Long term bath (3) Spraying	Suitable for large quantities of small fish (<5 g) Cost effective for small fish (1 and 3) Protective immunity for 3-5 months depending on the antigens	Difficult to apply on ongrowing units Expensive for large fish Costly (2)
Injection vaccination I.P. or I.M.	Good protective immunity, lasting up to 1 year Suitable for large fish (broodstock) Possibility of automatization Good for weak antigens, for vaccination at low temperature	Stressful method Labour and time consuming Use of anaesthetic required Side lesions
Oral vaccination	No stress, not time consuming Easy to apply on all production facilities For all sizes of fish (>10 g) and large batches	Monovalent vaccines only Protection slightly shorter than injection (8 months) Needs to be planned and requires good feeding practice

1.8 Probiotics

The first definition of probiotics was by Parker (1974) who defined them as "organisms and substances which contribute to intestinal microbial balance". Fuller (1989) developed this definition as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Recently, the following modified definition has been proposed, which allows a broader application of the term "probiotic", i.e. "A live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment" (Verschuere *et al.,* 2000).

Probiotics have been used in agriculture and human medicine for >20 years; they are comprised of live microbial cultures administered with the primary aim of preventing infectious diseases by strengthening the disease-barrier and/or by nonspecific enhancement of the host immune system (Fuller, 1997). To date, many probiotics have been developed for use in aquaculture (Austin & Day, 1990; Kennedy *et al*., 1998; De Schrijver & Ollevier, 2000; Robertson *et al*., 2000; Brunt & Austin, 2005).

Probiotics may produce specific compounds, including bacteriocins or bacteriostatic peptides (Klaenhammer, 1988), that inhibit growth of fish pathogens. For example, *Lactobacillus* produces inhibitory compounds with effectiveness against *A. hydrophila* (Lewus *et al*., 1991; Santos *et al*., 1996). Probiotics have been incorporated into the diet of shrimp to control the microflora of tanks (Garriques & Arevalo, 1995). Gildberg *et al*. (1995) found that probiotic bacteria, which were isolated from fish, can inhibit the growth of fish pathogens such as *V. anguillarum* and *A. salmonicida*. Also, *Vibrio alginolyticus* plays a probiotic effect in reducing diseases in Atlantic salmon (*Salmo salar*) caused by *A. salmonicida*, and *V. anguillarum*, (Austin *et al*., 1995). Moreover, Olsson *et al*. (1992) found that bacterial strains associated with intestinal and skin mucus of adult marine turbot (*Scophthalmus maximus*) and dab (*Limanda limanda*) have the ability to suppress the growth of *V. anguillarum*. As a further example, 5 probiotics isolated from the clownfish, *Amphiprion percula*, were examined for their ability to attach to fish intestinal mucus and compete with *A. hydrophila* and *V. alginolyticus*. The results showed a reduction in pathogen attachment (Vine *et al*.,

2004). Furthermore, a culture of *A. sobria* was effective when used as a probiotic insofar as it led to reduced mortalities among rainbow trout, *Oncorhynchus mykiss,* after challenge intraperitoneally with *Lactococcus garvieae* and *Streptococcus iniae* (Brunt & Austin, 2005). Here, the mode of action reflected stimulation of innate immunity (Brunt & Austin, 2005).

Interestingly, Moriarty (1998) suggested that bacterial mixtures distributed by water as "water additives" instead of "feed additives" may also have a beneficial effect on aquacultural production. Indeed, Queiroz & Boyd (1998) reported that a commercially prepared bacterial mixture of *Bacillus* spp. mixed into rearing water increased survival and production of channel catfish (*Ictalurus punctatus*). The question is whether or not these constitute *bona fide* probiotics or should they be regarded as methods of water disinfection.

1.9 Immunostimulants

By definition, "an immunostimulant is a chemical, drug, stressor or action that enhances the innate or non-specific immune response by interacting directly with cells of the system activating them" (Galindo-Villegas & Hosokawa, 2004). In practice, "immunostimulants are promising dietary supplements to potentially aid in disease control of several organisms including marine fish and increase disease resistance by causing up regulation of host defense mechanisms against opportunistic pathogen microorganisms in the environment" (Galindo-Villegas & Hosokawa, 2004). Immunostimulants increase resistance to infectious diseases, not only stimulating the acquired immune response, but also enhancing innate, humoral and cellular defense mechanisms (Galindo-Villegas & Hosokawa, 2004). Immunostimulants are considered safe and more environmental friendly than chemotherapeutics in addition to their wider efficacy (Sakai, 1999). It is relevant to note that Anderson (1992) suggested that immunostimulants should be applied before the outbreak of disease to reduce disease-related losses.

1.9.1 Types of immunostimulants

Many substances have proven effectiveness in enhancing the immune response of fish. Anderson (1992) grouped these compounds according to their main action on the immune system, i.e. cell stimulator or inflammatory agent, whereas Galeotti (1998)

grouped them according to their origin, namely synthetic chemical, bacterial derivatives, nutritional factors, animal derivatives, plant derivatives and hormones (Table 1.5).

Table 1.5 Immunostimulants used in fish (adapted from Sakai, 1999 and Galeotti, 1998)

Synthetic chemical	Levamisole
	FK-565
	Fluoro-quindone
	Avridine
Biological substances	
(1) Bacterial derivatives	MDP (Muramyl dipeptide)
	ß-glucan
	Peptidoglucan (*Brevibacterium lactofermentum; Vibrio* sp.)
	FCA
	EF203
	LPS (lipopolysaccharide)
	Clostridium butyricum cells
	Achromobacter stenohalis cells
	Vibrio anguillarum cells (vibrio vaccine)
(2) Polysaccharides	Chitin
	Chitosan
	Lentinan
	Schizophyllan
	Oligosaccharide
(3) Nutritional factors	
Vitamins	Carotenes/carotenoids
	Vitamin A
	Vitamin D
	Vitamin E
	Vitamin C
	Vitamin B_6
	Vitamin B_{12}
(5) Hormones, cytokines and others	Lactoferrin
	Interferon

	Growth hormone
	Prolactin
(6) Animal extracts	*Ecteinascidia turhinuru* extract (ETE)
	Haliotis discus extract (HDe)
	Chitosan (from shrimp carapace)
	Fish extract (ISK)
	EF-203 (from chicken eggs)
	Firefly squid
(7) Plant extracts	*Quillaja saponin* (soap tree)
	Glycyrrhizin (licorice)
	Traditional Chinese medicine
	Laminaran (seaweed)

1.9.2 Synthetic chemicals

Levamisole is an antihelminthic used for the treatment of nematode infections in man and animals. Incidental observations suggested a state of enhanced resistance to various kinds of infection upon treatment. Thus, rainbow trout exposed to a bath containing 5, 10 and 25 μg/ml levamisole for 2 h showed resistant to *Y. ruckeri* (Ispir, 2009). Moreover, dietary intake of 250 mg levamisole/kg in common carp fingerlings increased their resistance to *A. hydrophila* when injected intraperitoneally. This was followed by the effectiveness of the 500 mg/kg treatment group and then the 100 mg/kg group in addition to enhancement in non-specific immunity (Maqsood *et al.*, 2009). Another example is FK-565 (heptanoyl-y-D-glutamyl-(L)-meso-diaminopimelyl-(D)-alanine), which is a peptide related to lactoyl tetrapeptide (FK-156). Kitao & Yoshida (1986) demonstrated that injection of FK-565 into rainbow trout increased their resistance to challenge with *A. salmonicida*.

1.9.3 Bacterial derivatives

Muramyl dipeptide (MDP)(N-acetyl-muramyl-L-alanyl-D-isoglutamine) is derived from *Mycobacterium.* Kodama *et al.* (1993) reported that intraperitoneal injection of rainbow trout with MDP–Lys increased phagocytic, respiratory burst and migration activities of kidney leucocytes and resistance of the fish to challenge with *A. salmonicida.*

LPS is a cell wall component of Gram-negative bacteria. LPS was effective in preventing disease caused by *A. hydrophila* and in stimulating the innate immune response of rainbow trout (Nya & Austin, 2010). Moreover, Selvaraj *et al*. (2005) stated that LPS administered through injection and bathing effectively stimulated the non-specific cellular and secondary immune response, and offered protection against *A. hydrophila* infection in carp. Furthermore, Nayak *et al*. (2008) reported an increase in lysozyme, total globulin level, myeloperoxidase and respiratory burst activities in *L. rohita* after intraperitoneal injection with LPS.

β-Glucans comprise a diverse group of polysaccharides of D-glucose monomers linked by β-glycosidic bonds. They occur mostly in the cell wall of baker's yeast, other fungi, and bacteria. Selvaraj *et al*. (2005) recorded the highest antibody titre against *A. hydrophila* following injection with β-glucan (100–1000 μg glucans/fish). In addition, rainbow trout showed increased levels of phagocytosis and oxidative radical production after feeding with glucan for 4 weeks (Volpatti *et al.,* 1998). Intraperitoneal injection of β-glucan prepared from cell walls of *Saccharomyces cereĺisiae* into Atlantic salmon resulted in increased resistance to *V. anguillarum, V. salmonicida* and *Y. ruckeri* (Robertsen *et al.,* 1990). Dietary supplementation with different concentrations of β -1, 3 glucan in large yellow croaker, *Pseudosciaena crocea,* led to a significant increase in serum lysozyme activity with an increase of dietary glucan. The phagocytosis and respiratory burst activity significantly decreased in fish fed the diet with high supplementation compared with lower doses (Ai *et al.,* 2007).

Freund's complete adjuvant (FCA) is a mineral oil adjuvant containing killed *Mycobacterium butyricum*. Rainbow trout injected with FCA showed increases in respiratory burst, phagocytic and NK cell activity of leucocytes and were protected against *V. anguillarum* infection (Kajita *et al*. 1992). Previously, Adams *et al*. (1988) reported that FCA-injected rainbow trout appeared to show increased protection against furunculosis, vibriosis and ERM.

V. anguillarum inactivated whole cell vaccine is the most successful vaccine for salmonid fish, and this efficacy is seen following administration by injection, oral dosing and immersion methods (Sakai, 1999). Moreover, rainbow trout immersed in *V. anguillarum* vaccine showed increased protection to *Streptococcus* infection, i.e. there

is evidence of herd immunity (Sakai *et al*., 1995a). In a similar demonstration of cross protection, Norqvist *et al*. (1989) showed that vaccination of rainbow trout with attenuated *V. anguillarum* induced protection against *A. salmonicida* challenge.

1.9.4 Polysaccharides

Chitin is a polysaccharide forming the principal component of crustacean and insect exoskeletons and the cell walls of certain fungi (Sakai**,** 1999). Rainbow trout injected with chitin showed stimulated macrophage activities and an increased resistance to *V. anguillarum* infection (Sakai *et al*., 1992). In addition, yellowtail showed increased protection against *Photobacterium damselae* subsp. *piscicida* when injected with chitin, with the resistance continuing for 45 days (Kawakami *et al*., 1998).

Brook trout, *Salvelinus fontinalis*, injected with or immersed in chitosan solution (de-*N*-acetylated chitin) showed increased protection against *A. salmonicida* infection (Anderson & Siwicki, 1994). Also, rainbow trout treated with chitosan by injection or immersion demonstrated increases in immunological parameters in the blood such as NBT, potential killing activity, myeloperoxidase and total immunoglobulin concentration (Anderson *et al.,* 1995).

1.9.5 Nutritional factors

In teleosts, phagocytic and respiratory burst activities were significantly enhanced by administering increasing doses of vitamin C in feed. Thus, higher levels of dietary vitamin C significantly enhanced protection against challenge with *A. hydrophila* (Tewary & Patra, 2008). Moreover, Blazer & Wolke (1984) showed that vitamin E deficiency in trout resulted in reduced protection against *Y. ruckeri*. It is relevant to note that in yellow croaker (*Pseudosciaena crocea*), the activities of serum lysozyme, alternative complement pathway, phagocytosis and respiratory burst activity of head kidney significantly increased with increasing dietary vitamin C. Challenge experiments with *V. harveyi* showed that fish fed diets supplemented with vitamin C had significantly lower cumulative mortality compared to the controls (Ai *et al.,* 2007).

1.9.6 Hormones and cytokines

Sakai *et al.* (1995b, c) reported that exogenous growth hormone given to rainbow trout increased the production of superoxide anion in leucocytes. The same group reported

that the addition of homogeneous prolactin to chum salmon, *Oncorhynchus keta*, induced lymphocyte mitogenic responses (Sakai *et al.*, 1996d). Similarly, prolactin increased the production of superoxide anion by leucocytes in rainbow trout (Sakai *et al.,* 1996c).

Lactoferrin, which consists of a single peptide chain with a molecular weight ~ 87,000 Da and possessing two Fe-binding sites per molecule, is widely distributed in the physiological fluids of mammals (Sakai, 1999). Nile tilapia (*Oreochromis nilcoticus*) showed significant resistance to *A. hydrophila* after feeding with 600 mg of lactoferrin/kg. There was an increase in non-specific and specific immune activity, i.e. lymphocyte count, respiratory burst, serum lysozyme and bacteriocidal activities against *A. hydrophila* with addition of lactoferrin to diets (El- Ashram & El-boshy, 2008).

1.9.7 Animal extracts

Eel injected with Ete showed enhanced phagocytosis and increased survival following challenge with *A. hydrophila* (Davis & Hayasaka, 1984). Rainbow trout injected with Hde showed enhanced phagocytic and NK cell activities, and increased survival against *V. anguillarum* infection (Sakai *et al*., 1991). Moreover, Siwicki *et al*. (1994) recorded production of superoxide anion, potential killing activities by macrophages and the lymphoblastic transformation of lymphocytes *in vitro* in rainbow trout after administration of heated extract from firefly squid, *Watasenia scintillans*.

1.9.8 Plant extracts

Medicinal plants have been used in traditional systems to treat many diseases (Bhadauria *et al*., 2002). Secondary metabolites produced by plants are organic chemicals of high structural diversity, which play different functions including chemotherapeutic, bacteriostatic, bacteriocidal and antimicrobial functions (Purohit & Mathur, 1999). Recently, extensive research has been initiated to determine the feasibility of using herbal medicines in fish disease management insofar as these products are often without side effects and are biodegradable. Besides, the raw materials are inexpensive, locally available, and can be easily prepared (Harikrishnan & Balasundaram, 2005).

Nile tilapia showed enhanced phagocytic activity after administration of *Astragalus* extract for one week (Yin *et al.,* 2006; Ardo *et al*., 2008). Also, carp fed *Astragalus* extract showed enhanced respiratory burst, phagocytic and lysozyme activities and enhanced survival compared to control fish following challenge with *A. hydrophila* (Yin *et al*., 2009). Similarly, Divyagnaneswari *et al*. (2007) recorded a significantly enhanced production of reactive oxygen radicals in tilapia and decreased percentage mortality following challenge with *A. hydrophila* after administration of water soluble fractions of *Solanum trilobatum.* In another example, ginger extract was found to be very effective in enhancing phagocytosis and extracellular burst activity of blood leucocytes in rainbow trout (Dugenci *et al.*, 2003). Furthermore, Yuan *et al.* (2007) recorded that carp fed with diets containing a mixture of *Astragalus membranaceus*, *Polygonum multiflorum, Isatis tinctoria* and *Glycyrrhiza glabra* at 0.5 and 1% for 30 days resulted in significantly increased phagocytosis, respiratory burst activity and total protein. Moreover, the respiratory burst and lysozyme activities were significantly increased in common carp and large yellow croaker fed a ration containing a mixture of *Astragalus membranaceus* and *Angelica sinensis* (Jian & Wu, 2003; 2004). On a similar theme, feeding *Labeo rohita* with *Allium sativum* and *Magnifera indica* showed enhancement in superoxide anion production, lysozyme activity and bacteriocidal activity in addition to reduction in mortalities after challenge with *A. hydrophila* (Sahu *et al*., 2007a, b).

1.9.9 Methods of administration

In aquaculture, there are three different methods that have been used to administer immunostimulants, namely injection, immersion and oral uptake. Injection and immersion methods are suitable for intensive aquaculture, and require the fish to be handled or in a small, confined space during the procedures (Galindo-Villegas & Hosokawa, 2004).

Injection is the most effective method especially for large fish (fish must be > 10 -15 g in weight). Indeed, many authors have reported protection against a range of pathogens. Thus, intraperitoneal injection of channel catfish with glucan showed an increase in phagocytic activity and a decrease in fish mortality following challenge with *Edwardsiella ictaluri* (Chen & Ainsworth 1992). However, this method is labour intensive, relatively time-consuming, stressful due to the use of anaesthesia and

becomes impractical when fish weigh < 15 g (Sakai, 1999; Galindo-Villegas & Hosokawa, 2004).

The efficacy of immersion has been demonstrated by several authors (e.g. Baba *et al.,* 1993; Jeney & Anderson, 1993b; Anderson *et al*., 1995; Galeotti *et al*., 1995). Thus, using immersion with levamisole, sea bass (*Dicentrarchus labrax*) showed an increase in circulating leukocytes, phagocytic rate and increase protection against *P. damselae* subsp. *piscicida* (Galeotti *et al*., 1995**).** In another example, rainbow trout immersed with glucan or chitosan showed increased protection against *A. salmonicida* for 3 days. However, this effect was absent after 14 days (Anderson *et al*., 1995). Therefore, accurate dilution, exposure time and levels of efficacy are not well defined, and caution is needed with immersion uptake (Galindo-Villegas & Hosokawa, 2004).

Oral administration is the only method economically suited to extensive aquaculture. It is non stressful and allows mass administration regardless of fish size (Sakai, 1999; Galindo-Villegas & Hosokawa, 2004). Sakai (1999) stated that oral administration of immunostimulants resulted in enhancement of leucocyte function and protection against infectious diseases, such as furunculosis, vibriosis and streptococcosis**.** Oral administration is a widely used method, which is sometimes more effective than immersion. Nikl *et al*. (1993) compared the adjuvant effects of glucan against *A. salmonicida* vaccine applied orally for 7 days and by immersion for 15 min with the outcome that there were not any adjuvant effects with the latter, but with the former instead.

1.9.10 Dosage and time of administration

The effective dosage and exposure time will be further complicated by different feeding strategies applied in culture facilities (Gannam & Schrock, 1999). Robertsen *et al*. (1990) found injection of Atlantic salmon, *Salmo salar*, with high dose of glucans, i.e. 100 mg/kg, led to an absence of protection for one week, but maximum benefit occurred after three to four weeks. Also, injection with a low dose, i.e. 2-10 mg/kg, led to protection only at 7 days. It was apparent that the chemiluminescent effects of phagocytic cells in rainbow trout were increased by injection at 0.1 and 0.5 mg/kg of levamisole, whereas a higher dose of 5 mg/kg did not lead to any benefit (Kajita *et al.,* 1990). In another example, *Clarias fuscus,* which was fed with diets containing 75 mg levamisole/kg dry diet, did not lead to any effect on the immune response whereas 300

mg levamisole/kg stimulated nonspecific defence mechanisms. Conversely, 600 mg levamisole/kg caused immunosuppression (Li *et al*., 2006). Interestingly, the effective dose may vary against different pathogens. Thus, Robertsen *et al*. (1990) found M-glucan caused a high survival in Atlantic salmon against *V. anguillarum* compared to *V. salmonicida* or *Y. ruckeri*.

Few investigations have been done on the effect of long term oral administration of immunostimulants. Juvenile rainbow trout fed for 28 days with 0.2 and 2 mg/kg of peptidoglycan derived from *Bifidobacterium thermophilum* were protected against challenge with *V. anguillarum*, whereas feeding for 56 days did not lead to any significant difference (Matsuo & Miyazono, 1993). Similarly, Yoshida *et al.* (1995) noticed an increase in the number of NBT-positive cells in African catfish after oral administration of glucan or oligosaccharide over 30 days, but not over 45 days. In turbot, long-term oral administration of glucan did not lead to a reduction in mortality after a challenge with a virulent culture of *V. anguillarum* (De Baulny *et al.,* 1996).

1.9.11 Evaluation methods for immunostimulants

There are two main procedures for evaluating the efficacy of an immunostimulant: (1) *in vivo*, namely protection tests against fish pathogens; (2) *in vitro*, namely the measurement of the efficiency of cellular and humoral immune mechanisms.

In vivo protection tests against fish pathogens are currently used with successful results (Table 1.6). However, knowledge of the immune system is limited for most fish species, and information on the mode of action of most immunostimulatory substances is even more restricted (Galindo-Villegas & Hosokawa, 2004). *In vitro* evaluation methods include testing the effects of substances on the immune system, which include preliminary *in vitro* and secondary *in vivo* studies. For this reason, in *vitro* tests should be performed before in *vivo* experiments in order to elucidate the basic mechanisms responsible for the protection (Galeotti, 1998). It is also possible to study the effects of an immunostimulant directly on target cells collected from fish in which observations may provide information on the type of stimulation caused on the target cells (Galeotti, 1998). *In vitro* evaluation is usually based on the following parameters: serum lysozyme, complement, total protein, total leucocyte count,

monocytes/lymphocyte/granulocyte count, antibody titres, phagocytosis, respiratory burst and leucocyte proliferation.

Table 1.6 Immunostimulants showing effectiveness against pathogens in fish (adapted from Sakai, 1999; Galeotti, 1998; Galindo-Villegas & Hosokawa, 2004)

Immunostimulant	**Fish**	**Resistance pathogen**	**Authors**
Levamisole	trout	*V. anguillarum*	Kajita *et al*. (1990)
	trout	*A. salmonicida*	Jeney & Anderson (1993b)
	carp	*A. hydrophila*	Baba *et al*. (1993)
	trout	*Y. ruckeri*	Ispir, (2009)
	carp	*A. hydrophila*	Maqsood *et al*. (2009)
	gilthead bream	*V. anguillarum*	Mulero *et al*. (1998)
FK-156	trout	*A. salmonicida*	Kitao & Yoshida (1986)
MDP	trout	*A. salmonicida*	Kodama *et al*. (1993)
LPS	trout	*A. hydrophila*	Nya & Austin (2010)
	carp	*A. hydrophila*	Selvaraj *et al*. (2005)
FCA	coho	*A. hydrophila* *A. salmonicida*	Olivier *et al*. (1985)
	trout	*A. salmonicida* *V. anguillarum* *Y. ruckeri*	Adams *et al*. (1988)
V. anguillarum bacterin	trout	*A. salmonicida*	Norqvist *et al.* (1989)
	trout	*Enterococcus seriolicida*	Sakai *et al.* (1995c)
C. butyricum	trout	*V. anguillarum*	Sakai *et al*. (1995a,b)
A. stenohalis	char	*A. salmonicida*	Kawahara *et al.* (1994)
Chitin	trout	*V. anguillarum*	Sakai *et al*. (1992)
Chitosan	trout	*A. salmonicida*	Siwicki *et al*. (1994)
Glucan	salmon	*V. anguillarum* *V. salmonicida* *Y. ruckeri*	Robertsen *et al*. (1990)
	salmon	*V. anguillarum* *V. salmonicida*	Raa *et al.* (1992)
	catfish	*E. ictaluri*	Chen & Ainsworth (1992)
	salmon	*A. salmonicida*	Rørstad *et al*. (1993)
	turbot	*V. anguillarum*	De Baulny *et al.* (1996)
	catfish	*V. anguillarum*	Duncan & Klesius (1996)
	salmon	*A. salmonicida*	Nikl *el al*. (1991)
	carp	*E. tarda*	Yano *et al*. (1989)
	blue gourami	*A. hydrophila*	Samuel *et al*. (1996)
Lactoferrin	Nile tilapia	*A. hydrophila*	El- Ashram & El- boshy, (2008)
Vitamin C	trout	*V. anguillarum*	Navarre & Halver (1989)
	catfish	*E. tarda*	Li & Lovell (1985)
	salmon	*V. salmonicida*	Erdal *et al.* (1991)

	salmon	*A. salmonicida*	Hardie *et al*. (1991)
	trout	*I. multifiliis*	Wahli *et al*. (1995)
	salmon	*A. salmonicida*	Hardie *et al*. (1990)
	carp	*A. hydrophila*	Gopalakannan & Arul (2006)
Vitamin E	salmon	*A. salmonicida*	Thompson *et al*. (1994)
ETE (*E. turbinate*)	eel	*A. salmonicida*	Sigel *et al.* (1983)
HDe (*Haliotis Discus hannai*)	trout	*A. anguillarum*	Sakai *et al*. (1991)
ISK (polypeptide from fish)	trout	*A. salmonicida*	Jeney & Anderson (1993b)
EF-203 from chicken eggs	trout	*A. salmonicida*	Sakai *et al*. (1995e)
Astragalus extract	carp	*A. hydrophila*	Yin *et al.* (2009)
	yellow croaker	*V. alginolyticus*	Jian & Wu, (2003)
Allium sativum	*L. rohita*	*A. hydrophila*	Sahu *et al.* (2007b)
	trout		Nya &Austin (2009a)
Magnifera indica	*L. rohita*	*A. hydrophila*	Sahu *et al.* (2007a)
Solanum trilobatum	tilapia	*A. hydrophila*	Divyagnaneswari *et al.* (2007)
Glycyrrhizin	yellowtail	*E. seriolicida*	Edahiro *et al*. (1990)

1.10 Immunity in fish

It is customary to consider the immune system as the innate (= non-specific) and the acquired (= specific) immune system. Ellis (1999) described the immune defence mechanisms of fish against bacterial pathogens (Table 1.7). Although innate and adaptive immunity are often considered separately to facilitate their understanding, it is important to recognize that they frequently work together. For example, macrophages are phagocytic but produce important cytokines that help to induce the adaptive immune response (Lydyard *et al*., 2004). Complement components of the innate immune system can be activated directly by microbes, but can also be activated by antibodies, molecules of the adaptive system (Lydyard *et al*., 2004).

1.10.1 Acquired (specific) immunity

Acquired (specific) immunity plays an important role in the protection against recurrent infections by generating memory cells (= cell-medicated immunity) and specific soluble and membrane-bound receptors (= humoral defense) such as T-cells and immunoglobulins (Ig), which allow for the fast and efficient elimination of the specific pathogens (Galindo-Villegas & Hosokawa, 2004). T-cell populations are divided into

cytotoxic T-cells (Tc) and helper T-cells (Th). Tc kills viruses and intracellular pathogens, whereas Th secretes cytokines that initiate and regulate various immune responses (Secombes *et al.*, 2005). Ig's are produced by B-cells, and have the same basic four polypeptide chain structure, i.e. two light chains and two heavy chains (Lydyard *et al*., 2004).

Table 1.7 Immune defence mechanisms in fish against bacteria (after Ellis, 1999)

Humoral factors	**Cell- mediated factors**
Non- specific	
Inhibitors	**Neutrophils**
Transferrin	Respiratory burst → (O_2^-, H_2O_2, OH^-
Antiproteases (α 1- antiprotease; α 2- macroglobulin)	Halide+H_2O_2 (MPO) hypohalite ions ⟶
Antibacterial peptides	Lysozyme
Lectins	
Lysins:	**Macrophages**
Proteases	Hydrolytic enzymes
Lysozyme	Respiratory burst
C-reactive protein	NO (+O_2^- → peroxynitrite →OH^-)
Complement	
	Macrophage/Neutrophil cooperation
Specific	
Antibody	**Activated macrophages:**
Anti-adhesins	Specific T lymphocytes and antigen
Anti-toxins	↓
Anti-invasins	Cytokines (IFNɣ, TNF)
Activates classical complement pathway	↓
	Activate macrophages (enhanced Respiratory burst, enhanced bacteriocidal activity)

Fish antibodies are of much lower affinity and diversity than those of higher vertebrates, such as birds and mammals (Wilson & Warr, 1992). The cartilaginous fish (sharks, rays, skates, and chimaeras) and osteichthyan (bony) fish possess, as do all vertebrates except the agnathans, a high molecular weight polymeric IgM. In addition, some (but not all) fish possess low-molecular weight Ig (Warr, 1995). Ig possessed by fish are predominantly of four distinct structural types; a monomeric form of IgM (Wilson & Warr, 1992), a smaller form of monomeric IgM that can be designated IgM (Clem, 1971), a molecule termed IgX or IgR that is typical of the rajiformes and primitive sharks (Anderson *et al*., 1994; Kobayashi *et al*., 1992), and a molecule that has been termed IgN and may be IgY, in the lungfish (Marchalonis, 1969; Fellah *et al*., 1993). Recently, a novel isotype, namely IgZ or IgT, IgM–IgZ chimera molecule, and IgD and IgZ have been identified from zebrafish, rainbow trout, common carp and mandarin fish (Danilova *et al*., 2005; Hansen *et al.,* 2005; Savan *et al.,* 2005; Tian *et al.,* 2009).

1.10.2 Cytokines

Cytokines is derived from the Greek name meaning: cyto = cell and kinos = movement. Cytokines are regulatory proteins secreted by immune cells that initiate and regulate cellular function (Thomson, 1994). They exert a vital role in homeostatic mechanisms, such as the immune response, inflammation, acute phase response and tissue repair (Ollier, 2004). The level of a cytokine is a primary cause for a disease (Ollier, 2004). Specifically, cytokines include lymphokines (cytokines produced by lymphocytes), monokines (cytokines made by monocytes) and interleukins (cytokines made by one leucocyte and acting on other leucocytes). Recently, a number of fish cytokine genes has been isolated and sequenced, including those for transforming the growth factor-ß (Hardie *et al.,* 1998; Johnsona *et al.,* 2006; Haddad *et al.,* 2008), interleukin-1ß (Secombes *et al.*, 1997; Fujiki *et al.,* 1998; Bird *et al.,* 2002; Hong *et al.,* 2004; Corripio-Miyar *et al.,* 2006), and interleukin -8 (Laing *et al.,* 2002; Corripio-Miyar *et al.,* 2006; Abdelkhalek *et al.,* 2009).

1.10.3 Innate (non-specific) immunity

Innate immunity is the first line of defence against pathogen attack, and forms a vital role in preventing the establishment of infection in addition to the role in activating the acquired immune response. By definition, the innate immune system's recognition of non-self is mediated by germline-encoded pattern recognition proteins/receptors that

identify molecular patterns, which are characteristic of microbes (Magnadóttir, 2006). These molecules include polysaccharides, LPS, peptidoglycans, bacterial DNA and double stranded viral RNA (Medzhitov & Janeway, 1998; 2002; Elward & Gasque, 2003).

1.10.4 The main parameters of the innate immune system

The innate immune parameters have been studied in fish, both with respect to practical immunoprophylactic measures and in comparative or evolutionary immunology (Magnadóttir, 2006). Most of the parameters of the innate immune system of fish are shared by both invertebrates and vertebrates. The parameters are commonly divided into physical parameters, and cellular and humoral parameters (both can be cell associated receptors or soluble molecules of plasma and other body fluids).

1.10.5 Physical parameters

The first barrier in fish against infection is the scales, and mucus surfaces of skin and gills, and the epidermis (Ingram, 1980**;** Ellis, 2001). Several studies have emphasised that mucus is the most important defence in fish (Rombout *et al*., 1993; Rombout & Joosten, 1998; Fast *et al*., 2002). In particular, mucus plays a role in entrapment of pathogens (Austin & Mcintosh, 1988) by using immune parameters such as lectins, pentraxins, lysozyme, complement proteins, antibacterial peptides and Ig (Alexander & Ingram, 1992; Rombout *et al.*, 1993; Aranishi & Nakane, 1997).

1.10.6 The cellular parameters

Varieties of leukocyte types are involved in innate cellular defense of fish, including monocytes/macrophages, granulocytes and nonspecific cytotoxic cells. Phagocytic cells, mainly neutrophils and macrophages, play an important role in antibacterial defences. Secombes (1996), stated that the phagocytic cells can engulf bacteria and kill them by production of reactive oxygen species (ROS) during the process called respiratory burst. During this process, many oxygen radicals are released including superoxide anion (O_2), H_2O_2 and the hydroxyl free radical (OH^-), which have potent bacteriocidal activity. In addition, neutrophils contain myeloperoxidase (MPO) in their cytoplasmic granules (Afonso *et al.*, 1997), which can kill bacteria and produce bacteriocidal hypohalite ions in the presence of halide ions and H_2O_2 (Klebanoff & Clark, 1978). Additionally,

neutrophils and macrophages contain lysozyme and other hydrolytic enzymes in their lysosomes (Ellis, 1999).

1.10.7 The humoral parameters

Humoral parameters are classified commonly on the result of their pattern recognition specificities or effector functions (Magnadóttir, 2006**).** Ellis (1999**)** classified humoral parameters according to function on inhibitors (transferrin, antiproteases, antibacterial peptides and lectins) and lysins (proteases, lysozyme, C-reactive protein and complement).

Transferrin is an iron-binding glycoprotein, which acts as a growth inhibitor of bacteria by chelating the iron essential for bacterial maintenance (Langston *et al*., 1998). Also, it plays a role during the inflammatory response by removing iron from damaged tissue in addition to a role as an activator of fish macrophages (Stafford *et al*., 2001; Stafford & Belosevic, 2003). Yano (1996) stated that the amount of transferrin in host blood is important in deducing the condition of a pathogen-susceptible host. Transferrin expression was found to decrease in the liver of sea bass fish and increase in the brain in response to infection with *Photobacterium damselae* (Neves *et al.,* 2009)

Fish contain various protease inhibitors, principally α-1 antiprotease and α-2 macroglobulin, which are present in serum and other body fluids (Bowden *et al.*, 1997**;** Aranishi, 1999). The main role of these inhibitors is homeostasis of the body fluids. Also, they secrete proteolytic enzymes which are used in defence against pathogens (Ellis, 1987**;** Salte *et al.,* 1993; Zuo & Woo, 1997). Of relevance, Freedman (1991) recorded a difference in α-2 macroglobulin activity between rainbow trout and brook trout correlating with their resistance to *A. salmonicida* infection. Thus, a suggestion was made that α-2 macroglobulin may play a role in defence against furunculosis. Rainbow trout fed with probiotic showed enhancement in antiproteases and resistance against *Vibrio anguillarum* (Sharifuzzaman & Austin, 2009)

Lectins are proteins, which are able to bind to different carbohydrates, leading to opsonization, phagocytosis and activation of the complement system (Arason, 1996). In particular, chinook salmon egg lectin was inhibitory to growth of *A. hydrophila*, *V. anguillarum, E. tarda,* and *Y. ruckeri* (Voss *et al*., 1978). In parallel, lectin from coho

salmon egg agglutinated *A. salmonicida* cells, but not so any other fish pathogens including *A. hydrophila, V. anguillarum, V. ordalii* or *Renibacterium salmoninarum* (Yousif *et al.*, 1994a). Additionally, serum lectin of catfish significantly altered the viability and pathogenicity of the *Aeromonas* sp. through binding to bacterial cell wall resulted in a dose dependent increase in the bactericidal activity of fish macrophages (Duttaa *et al.,* 2005)

Anti-bacterial peptides are peptides with low molecular weights which have the ability to kill pathogens by disrupting the bacterial membranes (Ellis, 1999). Recently, they have been isolated from fish skin secretions (Lemaitre *et al*., 1996; Cole *et al*., 1997; Smith *et al.*, 2000; Narvaez *et al.,* 2010) and from fish tissue (Jiravanichpaisal *et al.,* 2007; Yue *et al.,* 2010)

Lysozyme is a lytic enzyme that plays an important role in preventing the invasion of microbes by splitting the ß(1-4) linkages between N-acetylmuramic acid and N-acetylglucosamine of bacterial cells thus resulting in lysis (Galindo-Villegas & Hosokawa, 2004; Magnadóttir, 2006). Also, lysozyme exerts a role in activating phagocytes and the complement system (Jolles & Jolles, 1984; Grinde, 1989). Lysozyme is found in mucus, lymphoid tissue, plasma and other body fluids of most fish species (Grinde *et al*., 1988; Lie *et al*., 1989; Yousif *et al.,* 1991; 1994b; Sun *et al.,* 2006; Larsen *et al.,* 2009). In particular, Murray & Fletcher (1976) identified lysozyme histochemically in monocytes and neutrophils of plaice.

C-reactive protein (CRP) binds to phosphorylcholine of the pathogen's cell wall in the presence of Ca ions (Magnadóttir, 2006). CRP plays a role in activating the complement system (Jiang *et al*., 1991; De Haas *et al.,* 2000) and in the recognition and clearance of apoptotic cells (Nauta *et al*., 2003). In rainbow trout, CRP activated complement and enhanced the phagocytosis and suppressed the growth of *V. anguillarum* (Nakanishi *et al.,* 1991). CRP has been isolated from fish in high quantity after exposure to temperature changes, chemical exposure or the administration of bacterial pathogens (Murai *et al*., 1990; Kodama *et al.,* 1989; Szalai *et al*., 1994; Kodama *et al.,* 2004).

Complement is an important part of the innate immune system, and contains ~35 soluble and membrane-bound proteins. The functions are numerous but include the

ability to kill pathogens by creating pores in their surface membranes. The killing mechanisms are activated either directly by the microorganisms or by antibody-antigen (Ag-Ig) complexes (Claire *et al.*, 2002). Additionally, complement plays an important role in inflammatory reactions by attracting phagocytic cells to the site of injury and simulating phagocytosis. Complement systems have three pathways: the classical complement pathway (CCP), the alternative complement pathway (ACP) and the lectin complement pathway (LCP). With the exception of jawless fishes, all pathways have been identified in fish (Fujii, *et al.,* 1992; Nonaka, 1994). ACP is directly activated by LPS in the cell walls of bacteria resulting in lysis (Ellis, 1999). The first pathway to be discovered was CCP, which is triggered by the binding of antibody to a cell surface (Claire *et al.*, 2002). LCP is initiated by binding of a complex protein consisting of mannose-binding lectin and serine proteases, and mannose-binding lectin associated proteases 1 and 2 to mannans on bacterial cell surfaces (Galindo-Villegas & Hosokawa, 2004).

1.11 Interaction between nutrition and immunity

Diet composition has an effect on immunity either by enhancing or retarding immunosuppression. However, many researchers realize that the food which provides the best disease resistance may not produce the fastest growth (Galindo-Villegas & Hosokawa, 2004). For example, feeding hybrid tilapia (*O. niloticus x O.aureus*) for 4 weeks with 0.5% garlic led to enhancement in immunity, namely leucocyte counts, respiratory burst, and phagocytic and lysozyme activity, but did not improve growth (Ndong & Fall, 2007).

1.12 Replacing fish meal with plant meal

Investigations have been carried out to evaluate the use of protein sources from plant products, such as seeds, leaves and other agricultural products (Olvera-Novoa *et al.*, 1988; El-Sayed, 1999; Makkar & Becker, 1999; Siddhuraju *et al*., 2000; Hossain *et al.*, 2001; Afuang *et al*., 2003; Richter *et al*., 2003). Studies have been done with salmonid diets in an effort to replace costly fish meal with other cheaper sources of plant protein, namely peas and faba beans (Gouveia *et al*., 1993; Pfeffer *et al.,* 1995), lupin (Gouveia *et al.,*1993; Bangoula *et al.,*1993; Glencross *et al*., 2004; Burel *et al*., 1998; 2000), rapeseed, canola (Bangoula *et al*.,1993; Stickney *et al.,* 1996) and other plants (Watanabe & Pongmaneerat 1993; Morales *et al*., 1994; Sanz *et al*., 1994). However,

the total replacement of fish meal with plant protein is not recommended as some authors reported a decrease in growth with increasing incorporation of plant meal (Fontainhas- fernandes *et al.*, 1999; Gomes *et al*., 1995; Glencross *et al*., 2004). These workers attributed the higher levels of crude fibre, the presence of protease inhibitors and anti-nutritional compounds in feeds with an effect on protein digestibility that leads to adverse physiological effects and reduction in growth (Olli *et al*., 1994; Vielma *et al*., 2000; Ali *et al*., 2003). In this connection, Soltan *et al.* (2008) observed that ≥45% replacement of fish meal with plant material led to significantly reduced feed intake, FCR and also negatively affected growth parameters (body length, weight gain and SGR) in Nile tilapia. Moreover, the total replacement of fish meal with a mixture of plant protein led to a marked decrease of feed intake and weight gain, whereas a 50-75% replacement produced only a slight reduction of growth rates (Sitjà-Bobadilla *et al.,* 2005).

Conversely, some studies have reported the lack of effect on growth and feed intake in Atlantic cod (*Gadus morhua*) when fed a mixture of different qualities of soybean meal and corn gluten of up to 68% of total protein and various soybean qualities of up to 24% of total protein (Albrektsen *et al*., 2006; Refstie *et al.,* 2006). Similarly, Espe *et al.* (2007) showed that close to 100% fish meal replacement is possible in diets for Atlantic salmon (*Salmo salar*) with no negative effect on growth if the amino acid profile in the feed is well balanced and feed intake is comparable to a high fish meal control diet. Also, Lee *et al.* (2002) revealed that fish meal could be entirely replaced by a mixture of plant proteins (cottonseed meal and soybean meal) and animal product proteins without adversely effecting growth rate and feed utilization in rainbow trout. Replacing fish meal with <50% of plant material does not appear to have a dramatic effect on fish growth. Thus, Fournier *et al.* (2004) found that replacement of up to 50% fish meal by lupin, corn gluten and wheat gluten meal in the diets of juvenile turbot (*Psetta maxima*) did not significantly affect growth rate, whereas 75 or 100% replacement significantly reduced growth rate. Nevertheless, tilapia and tilapia hybrids (*O. niloticus x O. aureus*) were observed to grow well when fed diets containing corn bi products and soybean meal as protein sources (Wu *et al.,* 1994; Twibell & Brown, 1998). Moreover, Jia *et al*. (1991) reported that inclusion of alfalfa in diets of Chinese blunt snout bream (*Megalobrama amblycephala*) resulted in increased feed intake and improvement of fish flesh. Certainly, Bilgin *et al.* (2007) observed that hazelnut meal could replace 20% and

30% of dietary soybean meal without growth suppressing effects on specific growth rate, relative growth rate, feed efficiency, and survival. In contrast, Glencross *et al*. (2004) recorded a decline in growth rate of rainbow trout when increasing yellow lupin level (0, 12.5, 25, 37.5 and 50%) in diets.

1.13 Aims of this study

The aims of the study are as follows:

1. The screening of different plants as dietary supplements and for their antibacterial activity against *A. hydrophila*, and the determination of the mode of action in rainbow trout.
2. Determination of the optimal dose of dietary supplements and the long term effects of feeding on immunological parameters.
3. Examination of the effect of long term feeding on enhancement of the adaptive immunity, e.g. cytokine genes.
4. Evaluation of the effects of dietary supplements on long term physiological factors, i.e. growth performance, and on biochemical and haematological parameters.
5. Examination of the effects of dietary supplements on digestive enzyme

Materials and Methods

2.1 Fish

Rainbow trout, average weight = 18 g, were obtained from commercial fish farms in Scotland, and acclimatized in aerated freshwater from a re-circulating system using biological filtration at a flow rate of 1-2 l/min for 14 days before use. During the acclimatization period, fish were fed three times daily with a commercial diet from Biomar (Aarhus, Denmark) (Table 2.1). The health status was examined immediately upon arrival in the aquaria (after Austin & Austin, 1989). Average water temperature ranged from approximately 8-14˚C, oxygen saturation was from 7-9 mg/l, and the pH was 6.0- 6.5. Photoperiod was adjusted at 14 h light and 10 h dark cycles.

2.2 Bacterial isolates

Aeromonas hydrophila (ORN2) were isolated from Koi carp, in England, and provided from the culture collection of the School of Life Sciences, Heriot-Watt University. Authenticity was verified after Austin & Austin (2007). Stock cultures were stored in tryptone soya broth (TSB; Oxoid, Basingstoke, U.K.) containing sterile 20% (v/v) glycerol at -70 ˚C. Cultures were routinely grown on tryptone soya agar (TSA; Oxoid) at room temperature for 48 h.

2.3 Bacterial identification by the API 20E system

The API 20E (Bio-Mérieux, Basingstoke, U.K.) was used, with incubation for 24 h at 27˚C. The manufacturers computer-based identification system was used to achieve identification.

2.4 Plants evaluated as immunostimulants

2.4.1 Lupin, *Lupinus perennis*

Dried lupin seeds were obtained from an Edinburgh supermarket, and ground using a household blender. Lupin powder was mixed with commercial fish food (Biomar, Table 2.1) to achieve 0.5 g (0.5%), 1 g (1%) and 2 g (2%) lupin/100 g of feed.

2.4.2 Mango, *Mangifera indica*

Fresh Mango fruit was obtained from an Edinburgh supermarket and washed and left to dry in an oven at 60˚C for 2 days, before grinding with a household blender to form a powder. This was mixed with commercial diet (Biomar) to achieve 0.5 g (0.5%), 1 g (1%) and 2 g (2%) mango/100 g of feed.

2.4.3 Stinging nettle, *Urtica dioica*

Dried leaves of stinging nettle (Good n' Natural, USA) were obtained from a health food shop in Edinburgh. A powder was prepared using a mortar and pestle, and mixed with commercial diet (Biomar) at the to achieve 0.5 g (0.5%), 1 g (1%) and 2 g (2%) stinging nettle/100 g of feed.

2.4.4 Other plant materials

Aloe vera, dong quai (*Angelica sinensis*), Astragalus root (*Radix astraglin*), *Echinacea purpurea* (extracts), and black paper were obtained from various outlets in Edinburgh, and mixed with commercial diets (Biomar) to achieve doses of 1g (1%)/100 g of fish feed.

2.5 Evaluation of the plants in rainbow trout

Rainbow trout were fed with feed supplemented with the plant material for 14 days. During the experiments, fish were observed for appetite. Immunostimulatory activity was evaluated by challenge with *A. hydrophila.* For challenge experiment fish were distributed into groups each contain 60 fish (20 per replicate).

Table 2.1 Composition of Biomar diet.

composition	**%**
Protein	53
Oil	19
Ash	10
Moisture	7.5
Phosphorus	1.2
Nitrogen	8.4
N.F.E.	10.5

2.6 Bacterial culture and challenge experiments

A. hydrophila was grown in nutrient broth (Oxoid) for 24 h at 37˚C. The culture was centrifuged at 3000 x *g* for 10 min at 4˚C, before the supernatants were discarded, and the pellets resuspended in 0.9% (w/v) saline. The bacterial suspensions were counted using a haemocytometer slide (Improved Neubauer type; Merck, Lutterworth, U.K.) at a magnification of x 400 on a Kyowa (Tokyo, Japan) light microscope. Challenge was by intraperitoneal injection with 0.1 ml volumes containing 10^7 cells/ml. Mortalities were recorded for 10 days, whereupon the survivors were examined pathologically after Austin & Austin (1989).

2.7 Cellular immune responses induced by the plant materials

Rainbow trout were distributed randomly into 9 groups each with 60 fish. The fish were fed for 14 days with 0.5%, 1% and 2% of lupin, mango and stinging nettle, and commercial diet only as controls, to examine the possible mode of action and effect on immune parameters. Blood was collected from fish (killed by an overdose of anaesthetic, i.e. 3- amino benzoic acid ethyl ester; Sigma-Aldrich, Basingstoke, U.K.) by venepuncture using 9 ml capacity before transferred to Vacuettes containing heparin (Greiner, Stonehouse, U.K.) to examine haematological parameters and blood cell differentiation. Blood was collected in Vacuettes without heparin and left to clot for 2 h at 4^{o}C before centrifuging at 3000 rpm for 25 min at 4^{o}C. Serum was collected, and stored at - 70 ^{o}C until use.

2.7.1 Haematological parameters

The blood was immediately used to determine the number of red blood cells (RBC) and white blood cells (WBC) by means of a haemocytometer slide (Improved Neubauer type) at a magnification of x 400. Thus, blood was diluted to 10^{-2} and 10^{-3} in PBS at pH 7.2 (Sarder *et al.,* 2001). Haematocrit (Hct) was determined by the microhematocrit method described by Brown (1988). Haemoglobin (Hb) concentration was conducted by using the cyanohaemoglobin method (Azizoglu & Cengizler, 1996). Briefly, 20 µl of whole blood was mixed with 5 ml of Drabkin's solution (Sigma-Aldrich) in a test tube before allowing to stand for at least 15 min at room temperature. The absorbance (A) was measured at 540 nm. The haemoglobin concentration of the blood sample was calculated from a curve prepared from known standards.

2.7.2 Blood cell differentiation

To differentiate blood cell type, blood films from triplicate samples were prepared on clean microscope slides before fixation for 5 min in 96% methanol and left to air dry. The slides were stained with Giemsa's stain for 20 min according to (Sarder *et al.,* 2001) and examined at a magnification of x 400.

2.7.3 Isolation of head kidney leucocytes

Isolation of head kidney macrophages was performed for the evaluation of phagocytic and respiratory burst activities. Rainbow trout were fed separately with lupin, mango, and stinging nettle for 14 days before being sacrificed by administration of an overdose of anaesthetic (3- amino benzoic acid ethyl ester; Sigma-Aldrich). The head kidney was isolated acorroding to Secombes (1990). Thus, head kidneys were removed, pooled, and forced through 100 µm nylon mesh with L-15 (Sigma-Aldrich) containing 100 µl/ml penicillin/streptomycin (p/s; 10 000 IU/ml/ 10 000 UG; Sigma-Aldrich), 10 µl/ml heparin (Sigma-Aldrich) and 2% (v/v) foetal calf serum (FCS; Sigma-Aldrich). Macrophage cells were layered onto 34/51% (v/v) Percoll (Sigma-Aldrich) diluted in HBSS. Samples were centrifuged at 400 x *g* for 25 min at 4ºC. The macrophage cells were removed from the interface and washed twice with HBSS (centrifugation at 350 x *g* for 10 min at 4ºC) and mixed with a equal volume of 0.4% (w/v) trypan blue (Sigma-Aldrich), and the presence of dead cells indicated (at a magnification x 400) by a blue colour which developed intracellularly within 5 min. The remainder of the cell suspension was adjusted to 10^6 cells/ml using a haemocytometer slide in L-15 medium supplemented with 0.1% (v/v) FCS and 100 µl/ml penicillin/streptomycin.

2.7.4 Phagocytosis activity

The phagocytic activity of macrophages was determined by the method of Sakai *et al.* (1995d). Briefly, 1.0 ml of leucocyte suspension (10^6 cells/ml), obtained from 10 individual fish, was pipetted onto sterile glass microscope slides and incubated at room temperature for 1 h, and non adherent cells were removed by washing three times with L-15 medium. After that, 1.0 ml of a latex bead suspension (0.85 µm, 10^9 particles/ml, Sigma-Aldrich) supplemented with L-15 and 10% (v/v) rainbow trout serum was added to the slides and incubated for 2 h at room temperature. Non-phagocytosed beads were washed off with L-15, and allowed to air dry. Air-dried slides were fixed with absolute methanol for 1 min. and stained by Giemsa's method (Houwen, 2002) and 100 cells were counted microscopically at a magnification of x 400. Phagocytic activity (PA) was determined by the following equation:

$$PA = \frac{\text{Number of phagocytosing cells}}{\text{Number of total cells}} \times 100$$

2.7.5 Respiratory burst activity

Respiratory burst activity was determined by reduction of nitroblue tetrazolium (NBT) to formazan as a measure of super oxide anion $(O_2)^-$ following the method of Chung and Secombes (1988), with some modification. Thus, 100 µl of the cell suspension were added to each well of a 96 well microtitre plate (Nalge-Nunc, Hereford, U.K.) with a final cell concentration of 10^6 cells/ml. The plates were incubated at 20ºC, for 2 h to allow attachment of cells. Then, unattached cells were washed off three times with fresh L-15 medium. L-15 medium was then supplemented with NBT (1 mg/ml) and phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich; 1 µg/ml) dissolved in dimethyl sulphoxide (DMSO, Sigma-Aldrich) and 100 µl of this solution added to each well of the microtitre plate and incubated for 1 h at room temperature. The medium was carefully removed from the plate and NBT reduction fixed with 100% methanol for 10 min. The plate was then washed with 70% methanol, and left to air dry. A mixture of 120 µl of 2 M potassium hydroxide (KOH) and 140 µl dimethyl sulphoxide (DMSO, Sigma-Aldrich) was added to dissolve the resulting formazan blue crystals. The absorbance (A) was read in Dynatech spectrophotometer at 630 nm (Dügenci *et al*., 2003) using KOH and DMSO as blanks.

2.8 Humorol immune responses induced by plant materials

2.8.1 Alternative complement activity

Alternative complement activity was assayed following the procedure of Yano (1992) by using rabbit red blood cells (RaRBC) since it was previously demonstrated to be one of the mammalian blood cells that is a better activator of the lytic reaction. Briefly, RaRBC were washed and adjusted to 2 x 10^8 cell/ml in ethylene glycol tetraacetic acid-magnesium-gelatin veronal buffer (0.01 M). Exactly 100 µl of the RaRBC suspension was lysed with 3.4 ml of distilled water and the absorbance of the haemolysate was measured at 414 nm against distilled water to obtain the 100% lysis value. The test serum was appropriately diluted, and different volumes ranging from 0.1 to 0.25 ml were made up to 0.25 ml total volume before being allowed to react with 0.1 ml of RaRBC in test tubes. After incubation at 20ºC for 90 min with occasional shaking, 3.15 ml of a 0.9% (w/v) saline solution was added to each tube with centrifugation at 1600 x

g for 10 min at 4ºC. The absorbance (A) of supernatant was measured using a spectrophotometer (DU 640, Beckman Coulter, Brea, California, USA) at 414 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of serum added. The volume yielding 50% haemolysis was determined and used for calculating the complement activity of the sample (ACH50) as follows:

ACH50 value (units/ml) = 1/K x (reciprocal of the serum dilution) x 0.5

Where K is the amount of serum (ml) giving 50% lysis and 0.5 is the correction factor since this assay was performed on half scale of the original method.

2.8.2 Lysozyme activity

The turbidimetric assay for lysozyme was carried out according to Parry *et al.* (1965). Thus, serum (40 µl) was added to 2 ml of a suspension of *Micrococcus lysodeikticus* (0.2 mg/ml) in a 0.05 M sodium phosphate buffer (pH 6.2). The reaction was carried out at 25ºC and absorbance was measured at 530 nm after 0.5 and 4.5 min on a spectrophotometer. A unit of lysozyme activity was defined as the sample amount causing a decrease in absorbance of 0.001/min.

2.8.3 Bactericidal activity

Serum bactericidal activity was done following the procedure of Kajita *et al*. (1990). An equal volume (100 µl) of serum and bacterial suspension were mixed and incubated for 1 h at 25ºC. A blank control was also prepared by replacing serum with sterile PBS. The mixture was then diluted with sterile 0.05 M sodium phosphate buffer, PBS (pH 6.2) at a ratio of 1:10. The serum-bacterial mixture (100 µl) was plated onto nutrient agar plates with incubation for 24 h at 25 ºC before the number of colonies was counted.

2.8.4 Total protein content

To measure the total protein content of the sera, a Bradford assay was carried out using bovine serum albumin (BSA) as the standard. Thus, 2 mg/ml solution of BSA was prepared and serial dilutions made with PBS (Oxoid). One ml of Bradford reagent (Sigma-Aldrich) was added to 20 µl of each dilution and incubated at room temperature for 15 min. The absorbance of each sample was then read at 595 nm in order to produce the standard curve. Dilutions (1: 100) were then made of the sera preparations in PBS and 1 ml of Bradford reagent added to 20 µl of each serum dilution. After incubation, the absorbance of the unknown samples was taken and plotted onto the standard curve to obtain the total protein content of the sera (Bradford, 1976).

2.8.5 Albumin and globulin content

Albumin content was measured by using the Albumin kit (BioAssay Systems, Hayward, CA, USA). Thus serum was diluted two-fold in distilled water before 20 µl of diluted serum and standard were transferred into wells of 96-well microtitre plates in triplicate. Then, 200 µl of working reagent was added to each well and mixed gently and incubated for 5 min at room temperature. The absorbance was recorded and plotted onto the standard curve to obtain the albumin content. Globulin content was calculated by subtracting albumin values from serum total protein.

2.8.6 Serum antiproteases

The serum anti-trypsin activity was measured by established methods **(**Ellis, 1987; Lange *et al.,* 2001). Thus, 20 µl of standard trypsin solution (Sigma-Aldrich, 1000-2000 BAEE, 5 mg/ml) was incubated with 20 µl of serum for 10 min at 22ºC. Subsequently, 200 µl of 0.1 M PBS (PH 7.2) and 250 µl of 2% azocasein solution (20 mg/ml PBS) were added and incubated for 1 h at 22ºC. The reaction was then terminated with the addition of 500 µl of 10 % (v/v) trichloro acetic acid (TCA) and incubated for 30 min at 2^{o}C. The mixture was centrifuged at 6000 x *g* for 5 min and 100 µl of the supernatant was transferred to a 96 microwell flat bottom plate containing 100 µl of 1 N NaOH/well. The absorbance was read in the spectrophotometer at 410 nm, and the

percentage inhibition of trypsin activity was calculated by comparing with a 100% control sample, in which buffer replaced the serum. For a negative control, buffer replaced both serum and trypsin.

2.8.7 Myeloperoxidase content

The total myeloperoxidase content present in serum was measured according to Quade and Roth (1997). For this, 50 µl serum was diluted with 135 µl of Ca^{+2} and Mg^{+2} free HBSS (Sigma-Aldrich) in flat-bottomed 96-well microtitre plates. Then, 50 µl of 20 mM 3,3´,5,5´-tetramethylbenzidine hydrochloride (TMB, Sigma-Aldrich) and 5 mM H_2O_2 (Sigma-Aldrich) were added (both substrates of peroxidase). The colour-change reaction was stopped after 2 min by adding 50 µl of 4 M sulphuric acid (H_2SO_4). The absorbance was read at 450 nm in a fluorimeter. Standard samples without serum were also analysed.

2.9 Determination of the effective period to induce immunity

Experiments were carried out in order to determine the effective period to induce immunity to infections disease. The fish were divided into 7 groups each containing 60 fish which were fed for 7, 21 and 28 days. Challenge with *A. hydrophila* was done after the corresponding number of days, i.e. 7, 21 and 28 days. Also, humoral assays including, complement, lysozyme, protein, albumin, globulin, myeloperoxidase, bactericidal and antiproteases were carried out at the same times.

2.10 Effect of long term feeding with the plant materials

Fish were distributed randomly into 7 groups, each containing 60 fish, and fed for 2 months with the supplemented diets. The effect of long term feeding on the general physiological health of fish including growth performance, body composition, kidney and liver function, glucose, digestive enzymes, electrolyte and haematological parameters (see Section 2.7.1 and 2.7.2) were done.

2.11 Digestive enzyme assays

Fish were starved for 2 days prior to sampling. Then, the fish were killed by overdose of anaesthetic followed by immediate dissection. The stomach and whole intestines were removed and rinsed separately with cold distilled water, and homogenized using a electric homogenizer. The homogenate was centrifuged at 15000 x *g* for 20 min at 4°C before the supernatant was removed and stored at -80˚C until needed. The total protein content of the supernatant was determined using the Bradford assay (Bradford, 1976).

2.11.1 Total proteases

The total protease activity was determined after Walter (1984) using the casein hydrolysis method with different pH values. Five buffers were used, namely KCl–HCl 0.1 M (pH 1.5), glycine–HCl 0.2 M (3.0), citrate 0.1 M-phosphate 0.2 M (pH 7.0) and Tris–HCl 0.1 M (pH 9.0) (see Appendix). A mixture of 0.25 ml casein (1%, w/v in distilled water), 0.25 ml buffer and 0.1 ml of enzyme extract were incubated for 1 h at 37°C. The reaction was stopped by adding 0.6 ml of 8% (v/v) trichloroacetic acid. The mixture was kept for 1 h at 2°C and then centrifuged at 1800 x *g* for 10 min. The absorbance of the supernatant measured at 280 nm. For the blank, the extract was added to the tubes at the end of the incubation just before adding the trichloroacetic acid. L-tyrosine was used as a standard. One unit of total protease activity was defined as the quantity of enzyme that released one mmol of tyrosine/ml/ min.

2.11.2 Pepsin activity

Pepsin activity was estimated by using 2% of haemoglobin (Sigma-Aldrich) in 0.06 N HCl as substrate, according to Worthington (1993). For this, 100 µl of enzyme extract (= homogenate) in 0.01 N HCl and 500 µl of substrate were incubated for 10 min at 37°C. The reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid (TCA; Sigma-Aldrich) and left for 5 min before centrifuging for 5 min at 12000 x *g*. Absorbance was recorded at 280 nm. In the blank, TCA was added to the substrate prior to the addition of enzyme extract. Specific activity (U) was expressed as:

$$\frac{\text{A (supernatant) - A (blank) x 1000}}{\text{10 min x mg protein}}$$

2.11.3 Amylase activity

Amylase activity was evaluated according to Natalia *et al.* (2004) by using 1% starch in 20 mM sodium phosphate buffer (pH 6.9, containing 6.0 mM NaCl) as substrate. Thus, 0.5 ml of substrate was added to 0.5 ml of enzyme extract, and incubated for 3 min at 55°C. This was followed by the addition of 0.5 ml of dinitrosalicylic acid (Sigma-Aldrich) and incubation in a boiling water bath for 15 min. A_{540} was recorded. The amount of maltose released was determined from a standard curve prepared from maltose solution. One unit was defined as the quantity of enzyme that released one mmol of maltose in 1 min.

2.11.4 Lipase activity

Lipase activity was determined with a rapid colorimetric kit (BioAssay Systems). For this, 150 µl H_2O, and 150 µl calibrator were pipetted into wells of a clear bottom 96-well microtitre plate (Nalge-Nunc). Then, 10 µl samples and 140 µl of the Working Reagent was added to each well with brief mixing A_{412} was recorded after 10 min (OD_{10}min) and 20 min (OD_{20}min). The activity was calculated as follows:

$$\text{Lipase Activity} = \frac{OD_{20min} - OD_{10min}}{OD_{Calibrator} - OD_{H2O}} \times 735 \text{ (U/l)}$$

2.12 Growth performance

All fish were deprived of food for 24 h before weighing and sampling. Subgroups of 10 fish were taken in triplicate for each group, and the following parameters were measured:

Weight gain (g) = final body weight – initial body weight

Specific growth rate (SGRs) (%) =

$$\frac{\text{Final mean body weight – initial mean body weight (g)}}{\text{Time interval (days)}}$$

Condition factors (CFs) = $\frac{\text{Weight (g)}}{[\text{Length (cm)}]^3}$

Feed intake (g/fish) = $\frac{\text{Dry feed intake (g)}}{\text{Number of fish}}$

Feed conversion ratio (FCR) (%) = $\frac{\text{Feed intake (g)}}{\text{Weight gain (g)}}$

Daily intake rate (DIR) = ([feed intake /mean weight] / no. days) ×100

2.13 Body composition

At the end of feeding period, all fish were starved for 24 h before subgroups of 7 animals were sacrificed and dried intact in an oven at 105ºC for 24 h before the whole

body was crushed for body composition analysis according to AOAC guidelines (Association of Official Analytical Chemists, 1990).

2.13.1 Moisture content

Samples were weighed before maintaining in an oven for 24 h at 105°C, and reweighing to estimate the moisture content. The dry samples were crushed to a fine powder and preserved in a dissector for the determination of protein, fat and ash content.

$$\text{Moisture content (\%)} = \frac{\text{Weight after drying}}{\text{Weight before drying}} \times 100$$

2.13.2 Crude protein

Crude protein was determined by using a micro Kjeldahl method by measuring the total nitrogen content of the sample multiplied by the empirical factor 6.25. This method includes three stages;

1- Digestion by sulphuric acid

2- Distillation

3- Titration

% protein = % Nitrogen x 6.25

2.13.3 Crude lipid (diethyl ether extract)

Crude lipid content was determined by weighing the filter paper containing the dried sample and then transferring to Soxhlet apparatus using diethyl ether at 60-80°C for 12 h. The sample with the filter paper was dried and reweighed, the difference between sample weights indicating the total lipid content in the sample.

$$\text{Fat (\%)} = \frac{\text{Sample weight after fat extraction}}{\text{Sample weight before fat extraction}} \times 100$$

2.13.4 Ash content

Ash content was determined by weighing the crucible containing the dried sample and then transferring to a muffle furnace at 550°C for 8.0 h. The crucible containing the sample was reweighed and the difference between sample weights indicated the ash content.

$$\text{Ash (\%)} = \frac{\text{Sample weight after oven}}{\text{Sample weight before oven}} \times 100$$

2.14 Serum electrolyte

Electrolytes, i.e. sodium, potassium, calcium, magnesium, and iron ppm/ml were determined by flame emission photometry using an automated system- Atomic absorption spectrometer (Perkin Elmer Precisely Analyst 200) with appropriate standard. The instrument was calibrated by diluting the standard in Millipore water (according to the manufacturer's instructions) to reach a volume of 5, 2.5, 1.25, and 0.625 ppm. Triplicate serum samples were diluted in Millipore water before measurement.

2.15 Glucose

Glucose was assayed by using the Pointe Scientific, (Canton, MI, USA) system. Serum glucose was measured within 1 h of sampling. Thus, 1.0 ml of reagent (see Appendix) was mixed with 5 µl of serum (= sample), and 5 µl of standard (100 mg/dl of glucose) in separate tubes. The mixture was left to stand at room temperature for 3 min, before recording absorbance on a spectrophotometer at 340 nm against water (= blank). Glucose (mg/dl) was calculated, as follows:

$$= \frac{\text{OD sample} - \text{OD blank}}{\text{OD standard} - \text{OD blank}} \times \text{Standard concentration (g/dl)}$$

2.16 Liver function

2.16.1 Glutamic pyruvic transaminase (GPT)

GPT was determined by using a Thermo Fisher (Waltman, MA, USA) scientific kit. Briefly, 30 µl of sample was added to 300 µl of reagent (see Appendix). The reaction was left for 3 min and the absorbance measured at 340 nm. Activity was conducted as follows:

Activity in U/l = OD/min x Factor

$$\text{Factor} = \frac{TV \times 1000}{6.3 \times SV \times P}$$

Where:

TV= Total reaction volume in ml

SV = Sample volume in ml

6.3 = millimolar absorption coefficient of NADH at 340 nm

P = Cuvette path length in cm.

2.16.2 Glutamic oxaloacetic transaminase (GOT)

GOT was determined by using a Thermo Fisher scientific kit. Thus, 15 µl of sample was added to 240 µl of reagent **1** and 60 µl of reagent **2** (see Appendix). The absorbance of the mixture was measured at 340 nm, and activity was calculated as follows:

Activity in U/l = OD/min x Factor

$$\text{Factor} = \frac{TV \times 1000}{6.3 \times SV \times P}$$

Where:

TV= Total reaction volume in ml

SV = Sample volume in ml

6.3 = millimolar absorption coefficient of NADH at 340 nm

P = Cuvette path length in cm.

2.17 Kidney function

2.17.1 Creatinine

The creatinine assay was done by using a kit from BioAssay Systems. With this, the standard was diluted to 2 mg/dl by mixing 5 µl 50 mg/dl standard stock and 120 µl distilled water. Then 30 µl diluted standard and plasma were transferred in duplicate into wells of a clear bottom 96-well microtitre plate. The Working Reagent was prepared by mixing per well reaction at least 100 µl reagent A and 100 µl reagent B. Then 200 µl of the Working Reagent was added quickly to all wells, before the plate was tapped briefly to achieve mixing. The absorbance was recorded at 1 min (OD_1) and 5 min (OD_5) at 510 nm. The creatinine concentration of the sample was calculated as

$$= \frac{\text{OD sample 5} - \text{OD sample1}}{\text{OD standard5} - \text{OD standard1}} \times \text{Standard concentration (2 mg/dl)}$$

2.17.2 Urea

Serum urea was measured by using a kit from BioAssay Systems. Then, 5 µl water (= blank), 5 µl standard (50 mg/dl) and 5 µl samples were transferred in duplicate into wells of a clear bottom 96-well microtitre plate. Next, 200 µl working reagent (an equal volume of reagent A and reagent B) was added before incubating for 20 min at room temperature. A_{520} was recorded. The urea concentration (mg/dl) of the sample was calculated as:

$$= \frac{\text{OD sample} - \text{OD blank}}{\text{OD standard} - \text{OD blank}} \times \text{Standard concentration (mg/dl)}$$

2.19 Cytokine gene expression induced by plant materials

Fish were fed with 1% and 2% of lupin, mango and stinging nettle for 2 months, and with commercial diet as control. Head kidney macrophages were stimulated by intraperitoneal injection with 0.1 ml of a suspension of *Aeromonas hydrophila* containing 10^6 cells/ml. The fish were left for 4 days before sacrificing with an overdose of anaesthetic (3-amino benzoic acid ethyl ester; Sigma-Aldrich). Approximately 100 mg of head kidney was isolated from each fish, placed in Eppendorf tubes and immediately immersed in liquid nitrogen before storage in -80^{o}C until use.

2.19.1 RNA extraction and reverse transcription

RNA extraction was done by using Tri reagent (Sigma-Aldrich), as shown in Fig. 2.1. The product was run on a1% agarose-MOPS-formaldehyde gel. Briefly, 1 g agarose was added to 80 ml dH_2O and 10 ml 10x MOPS (see Appendix). 10 ml formaldyde was added to mix after oven and cold. The mixture was poured into a 5.7 cm x 8.3 cm gel (W x L), and the edges were sealed with autoclave tape. A comb with 8 or 14 wells was inserted into the gel to form the sample slots. After the gel was completely set, the comb was removed and enough 1x MOPS buffer was added to cover the gel. Heat 5-20 µg RNA with 2 vol of loading buffer (see appendix) at 65-70^{o}C for 10- 15 min and cool on ice. Spin briefly and loaded on the gel. Electophoresis was carried out for 30 min. at 150 V and photographs were taken using a gel documentation unit (Amersham Bioscience, Little Chalfont, U.K.).

Reverse transcription was done with a cDNA kit from Fermentas (York, UK). For this, 1 µg total RNA was added to 1 µl oligo $(dT)_{18}$ primer and make the volume up to 11 µl with DEPC-treated water. The mixture was incubated at 70^{o}C for 5 min and chill on ice. Then, 4 µl 5x reaction buffer, 1 µl RiboLock™ RNase Inhibitor and 2 µl 10 mM dNTP mix were added with incubation at 37^{o}C for 5 min, before 2 µl of M-MuLV reverse transcriptase was added with further incubation at 45^{o}C for 60 min. The reaction was stopped by heating at 70^{o}C for 10 min followed by chilling on ice. Finally, 20 µl of cDNA was diluted with distilled water and stored -20^{o}C.

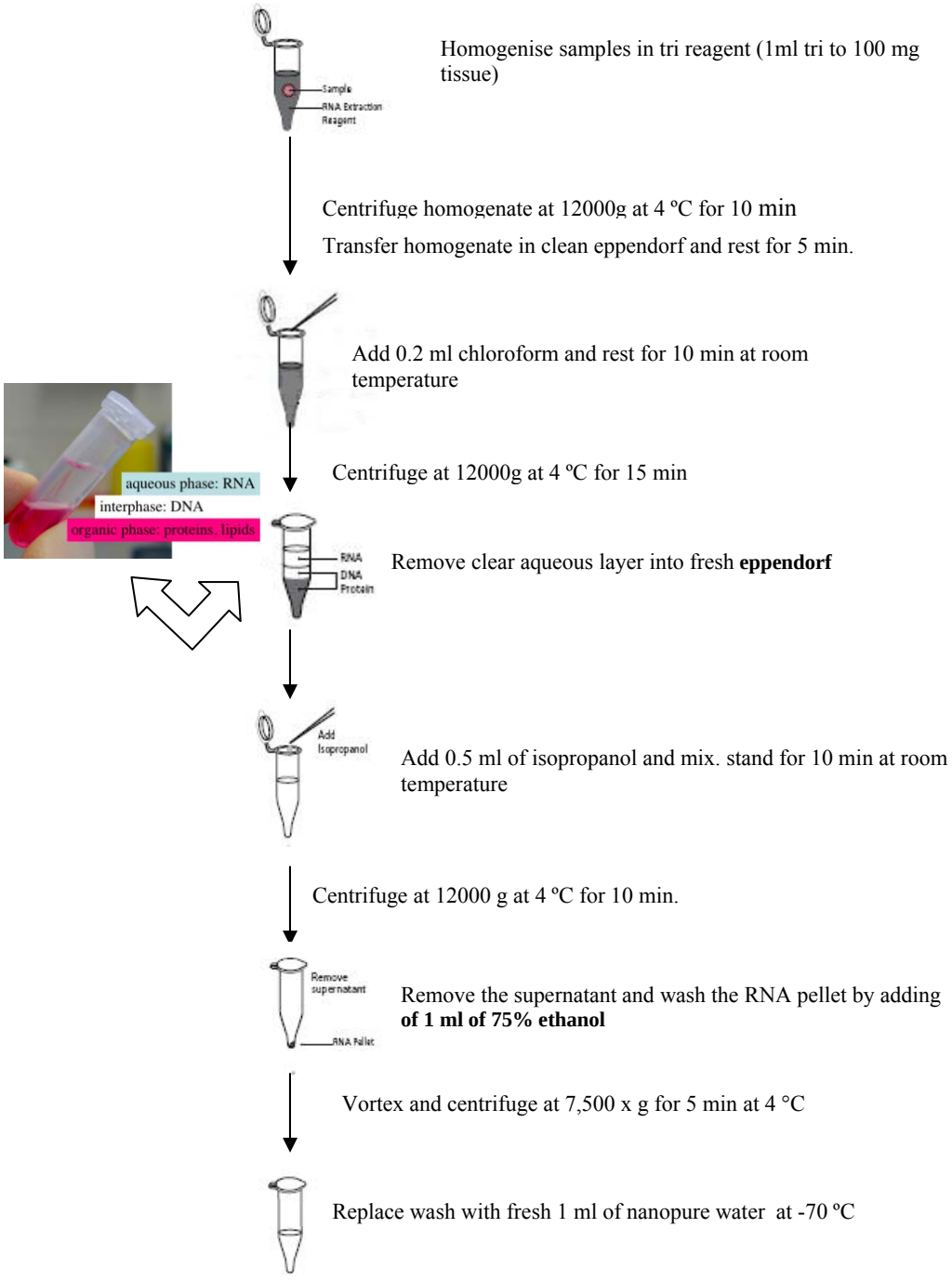


Fig. 2.1 Extraction of RNA from tissue

2.19.2 Primer design

Primers were chosen according to Kim & Austin (2006). They used Primer 3 software (Rozen & Shaleshy, 2000) in order to eliminate primer dimer formation and obtain the best for PCR with SYBR green fluorescence. Initial conditions for choosing the primers were as follows: amplification product size 100- 250 bp, primer size 20 ± 2 bp, GC content 50 ± 5 %, primer T_m 60 ± 2°C , max self complementarity = 3.0 and max 3' self-complementarity = 0 (Table 2.2)

2.19.3 Optimisation of PCR conditions

PCR was performed with each primer to optimise annealing temperature for the respective gene. The total volume was 50 µl, and comprised 5 µl 10x PCR buffer, 1 µl of dNTP mix (10 mm), 1 µl of forward and reverse primer (final concentration; 20 µM), 3µl of $MgCl_2$ (25 mM), 2 µl of cDNA, 1µl of *Taq* DNA polymerase (5u/µl) and sterile MilliQ (Millipore) water to achieve the final volume of 50 µl. PCR was carried out using Applied Biosystems machine with the following protocols; initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 20 sec, annealing at various temperature for each run (57, 58, 59, 60 and 61°C) for 30 sec, extension at 72°C for 20 sec, and a final extension at 72°C for 10 min. The annealing temperature was finally chosen after analysis of PCR products on 1% agarose gels.

2.19.4 Agarose gel electrophoresis of DNA

For examination of DNA fragments, 1% (w/v) agarose (Integra BioScience, Nottingham, U.K.) was dissolved in 1X of TAE (see Appendix) by microwave treatment and after cooling to 55°C ethidium bromide (Sigma-Aldrich) was added to a final concentration of 0.5 µg/ml. The mixture was poured into a 5.7 cm x 8.3 cm gel (W x L), and the edges were sealed with autoclave tape. A comb with 8 or 14 wells was inserted into the gel to form the sample slots. After the gel was completely set, the comb was removed and enough TAE buffer was added to cover the gel. A mixture of 5 µl of DNA and 6x gel-loading buffer was loaded into the slots of the submerged gel. A marker of 1 kps (Hyper ladder 1; Bioline, London, U.K.) was loaded in another well for

comparative molecular weight determination of the DNA. Electrophoresis was carried out for 2 h at 90V and photographs were taken using a gel documentation unit (Amersham Bioscience, Little Chalfont, U.K.).

2.19.5 Quantification of cytokine gene expression by real-time PCR

Real-time PCR, was done by using iQ SYBR Green Supermix (Bio-Rad, Hemel Hempstead, U.K.) following the manufacturer's instructions. The mixture comprised 25 µl of SYBR green mix, 2 µl of cDNA (~ 50 ng of total RNA) and each primer (final concentration: 150 nM) and Millipore water was added to a final volume of 50 µl. Real-time PCR was carried out using an iCycler^{TM} with the following protocols; enzyme activation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 20 sec, annealing at 58°C (for ß-actin and IL-8) or 61°C (for IL-1ß and TGF- ß) for 30 sec, and extension at 72°C for 30 sec. The PCR program was followed by a melt curve program with a heating rate of 0.5°C per sec (for 10 sec) and continuous fluorescence measurement.

2.19.6 Data analysis

The threshold cycle (C_T) was determined manually for each run. PCR efficiencies for each set of primers were determined using serial dilutions of cDNA (10 fold of each other) and resulting plots of C_T versus the logarithmic cDNA dilution, using the Efficiency equation (E):

$$E = 10^{(-1/\text{ slope})}$$

Gene expression go the samples compared to the controls were calculated according to the following equation, using REST © software 2009 (Pfaffli *et al.,* 2002).

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_{T\text{ target (control – sample)}}}}{(E_{\text{ß-actin}})^{\Delta C_{T\text{ target (control – sample)}}}}$$

Statistical analyses were done using the Pair Wise Fixed Reallocation Randomisation Test © in REST© software.

2.20 Statistical analyses

With the exception of real- time data, all data were analyzed by one-way analysis of variance (ANOVA). When differences were found among treatments, Tukey's test was used to compare means by Minitab statistical software (Minitab, Coventry, UK). Differences were considered significant at $P < 0.05$.

Table: 2.2 Sequences of oligonucleotide primers conditions for real-time PCR

Gene target	Primer name	Products size (bp)	Oligonucleotide (5'-3')	Factor-specific conditions	
				Annealing temp (°C)	Melting temp. (°C)
ß-actin	F	186	GGACTTTGAGCAGGAGATGG	58	91
	R		ATGATGGAGTTGTAGGTGGTCT		
IL-1ß	F	181	ACCGAGTTCAAGGACAAGGA	61	89
	R		CATTCATCAGGACCCAGCAC		
IL-8	F	162	CACAGACAGAGAAGGAAGGAAAG	58	82
	R		TGCTCATCTTGGGGTTACAGA		
TGF-ß1	F	275	AGATAAATCGGAGAGTTGCTGTG	61	89
	R		CCTGCTCCACCTTGTGTTGT		

Results

3.1 Bacterial identification by the API 20E system

The pathogen (ORN2) used in this study was confirmed as *Aeromonas hydrophila* by use of the API 20E rapid identification system (Table 3.1) with an excellent identification (probability of a correct identification of 99%) according to the manufacturer's database.

Table 3.1 Identification of ORN2 by API 20E rapid identification system.

Test	**Bacteria**
	ORN2
ß-galactosidase	+
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	-
Citrate utilization	-
Hydrogen sulphide production	-
Urease production	-
Tryptophan deaminase production	-
Indole production	+
Voges-Proskauer reaction	+
Gelatinase production	+
Production of acid from:	
Glucose	+
Mannose	+
Inositol	-
Sorbitol	-
Rhamnose	-
Saccharose	+
Melezitose	-
Amygdalin	+
Arabinose	-
Oxidase production	+

+ = positive reaction, - = negative reaction

3.2 Plants evaluated initially as dietary supplements

The primary results of eight plants or plant materials used as dietary supplements dosed at 1% in fish feed showed protection against challenge with *A. hydrophila* in the groups fed with lupin, mango and stinging nettle more so than the others (Table 3.2). Therefore, the results led to further experiments with these three plants.

Table 3.2 Preliminary test results of eight plant based products in terms of survival after challenge with *A. hydrophila.* The fish were fed with 1% of the plant material for 14 days prior to challenge.

Compound	Survival percent (%)
Lupin	100
Mango	96
Stinging nettle	96
Aloe vera	72
Dong quai (*Angelica sinensis*)	56
Astragalus root (*Radix astraglin*)	40
Echinacea purpurea (extract)	20
Black paper	0

3.3 Effect of plants on rainbow trout after challenge with *A. hydrophila*

Following use of 0.5% doses for 14 days, (Fig. 3.1) mango led to the highest survival (85%), ($p < 0.05$), followed by stinging nettle (70%), lupin (50%) and the control (48%). Whereas the differences were statistically significant between the controls and mango and stinging nettle, this was not the case with lupin ($p > 0.05$). With use of 1% doses, the control group (mortalities = 32%) showed the lowest survival ($p < 0.05$) whereas there was significant difference between control and the treatment groups which received the dietary supplements. At 2% doses, the controls again showed the lowest survival, i.e. 50% ($p < 0.05$). Use of mango led to the highest survival (80%) whereas lupin and stinging nettle resulted in 70% survival of the fish after challenge.

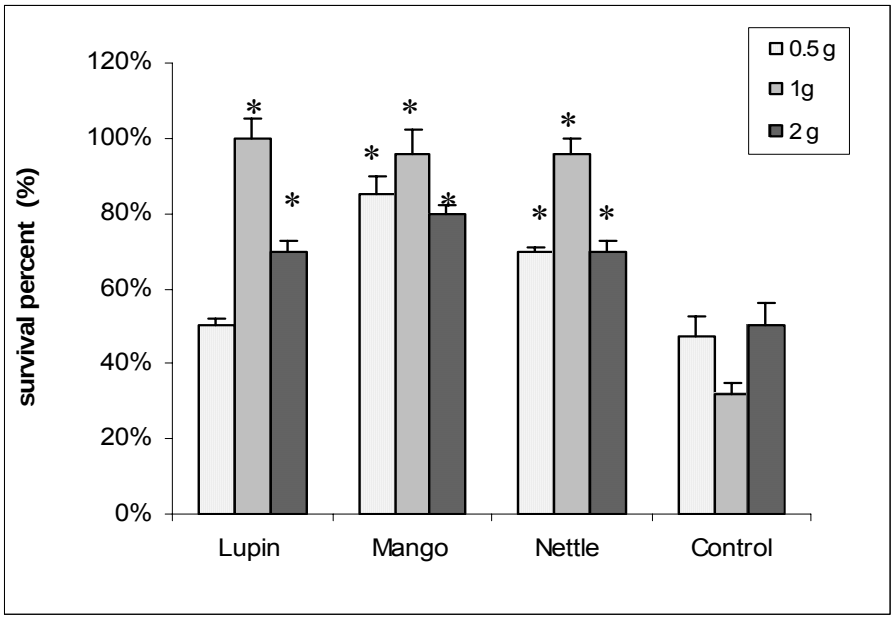


Fig. 3.1 Survival of rainbow trout fed for 14 days with 0.5%, 1% and 2% of lupin, mango and stinging nettle after challenge with *A. hydrophila*

* = $p < 0.05$

Bars = mean ± Standard Error (S.E.)

3.4 Induced cellular immune response after administration of dietary supplements for 14 days

3.4.1 Phagocytic activity

The highest phagocytic activity was in fish that received 1% doses of lupin (66%), mango (58%) and stinging nettle (63%), ($p < 0.05$), followed by activity in the 2% and 0.5% doses. The control showed the lowest activity at 20% (Fig. 3.2).

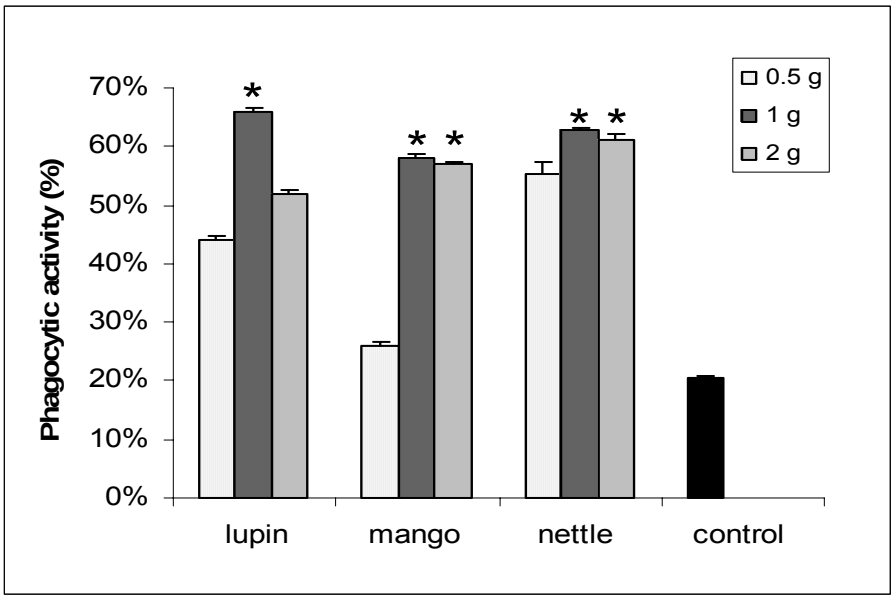


Fig. 3.2 Phagocytic activity of rainbow trout fed for 14 days with 0.5%, 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.4.2 Respiratory burst

With lupin, a highly significant difference occurred with the 1% dose (OD = 0.08), ($p < 0.05$) followed by 2% (OD = 0.06) and 0.5% (OD = 0.05) (Fig. 3.3), respectively. Also, 1% doses of mango (OD = 0.09) and stinging nettle (OD = 0.1) showed the highest value in respiratory burst (with highly significant difference between them and the control, $p < 0.05$), followed by the 2%, 0.5% and control doses, respectively.

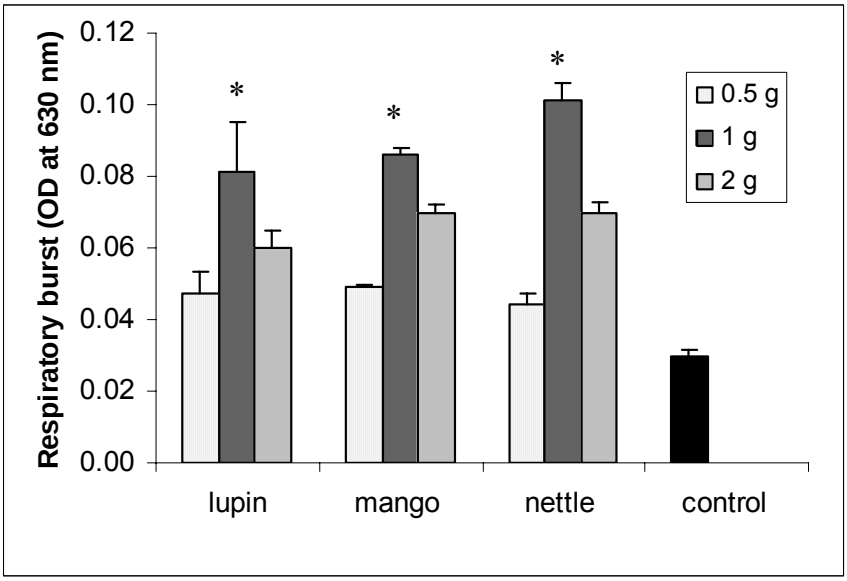


Fig. 3.3 Respiratory burst of rainbow trout fed for 14 days with 0.5%, 1% and 2% of lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.4.3 Red blood cell (RBC) and white blood cell (WBC) count

RBC and WBC (Table 3.3) counts were significantly higher in fish which were fed with 1% lupin ($p < 0.05$), followed by the 2% and then the 0.5% doses. The highest RBC count resulted from use of 1% mango, followed in turn by the 2% and 0.5% doses. In contrast, 2% stinging nettle showed the highest RBC count, followed by the 1% and 0.5% doses, respectively. Regarding the WBC count, 2% stinging nettle and 2% mango led to significantly higher values ($p < 0.05$) than the 0.5% dose and the control. Generally, controls showed the lowest value as compared with the treatment groups.

3.4.4 Haematocrit (Hct) and haemoglobin (Hb)

Hct was significantly higher than the controls ($p < 0.05$) following use of 2% lupin and 2% mango (Table 3.3). Conversely, 0.5% stinging nettle showed the highest values, followed by the 2% and 1% doses, respectively. Hb was higher in fish fed with 2% lupin, mango and stinging nettle, followed by the 1% doses and 0.5% dose in stinging nettle and mango, respectively. In terms of lupin, the highest level was recorded with the 2% dose followed by the 0.5% and 1% doses. There was no significant difference in Hb between any doses and the controls ($p > 0.05$).

Table 3.3 RBC, WBC, Hct and Hb of rainbow trout fed for 14 days with 0.5%, 1% and 2% of lupin, mango and stinging nettle.

	Dose	**RBC x 10^6/µl**	**WBC x 10^4/µl**	**Hct (%)**	**Hb (g/dl)**
	0.5%	1.53 ± 0.04*	7.58 ± 1.43	41.87 ± 0.36	12.46 ± 0.68
Lupin	1%	1.58 ± 0.11*	12.55 ±0.93*	38.76 ± 0.51	9.77 ± 0.47
	2%	1.54 ± 0.13	11.82 ± 1.31	47.10 ± 0.50*	13.51 ± 0.92
	0.5%	1.11 ± 0.20	6.72 ± 1.87	36.75 ± 0.20	11.54 ± 0.32
Mango	1%	1.48 ± 0.11	12.54 ± 2.66*	38.23 ± 2.14	11.60 ± 0.08
	2%	1.41 ± 0.15	11.88 ± 1.13	45.10 ± 1.57*	11.61 ± 1.59
	0.5%	1.25 ± 0.09	5.75 ± 3.63	40.73 ± 0.19	10.67 ± 0.26
Nettle	1%	1.27 ± 0.08	14.01 ± 0.38*	39.08 ± 0.31	11.44 ± 0.18
	2%	1.38 ± 0.05	9.23 ± 0.48	39.88 ± 0.79	12.74 ± 1.39
Control		1.09 ± 0.07	3.49 ± 0.79	31.79 ± 0.63	10.51 ± 0.86

Data represented as mean ± SE.

(*)= Significant difference from control $P < 0.05$.

3.4.5 Differential leucocyte and thrombocyte counts

Differential leucocyte and thrombocyte counts for lupin, mango and stinging nettle have been included in Table 3.4 and Fig. 3.4. Thus, the use of the 0.5% dose in lupin led to the highest lymphocyte number. Conversely, the use of 1% and 2% doses led to the highest significant number of monocytes ($p < 0.05$). The 1% dose resulted in higher numbers of neutrophils, followed by the 2% and 0.5 % doses, respectively. However, the highest lymphocyte count resulted with the 0.5% and 2% doses of mango, which was nevertheless lower than the controls. The 1% dose of mango gave the highest percent of monocytes ($p < 0.05$) and neutrophils. Thrombocytes were significantly higher in fish after feeding with 0.5% mango ($p < 0.05$) as compared to the controls, and followed by the 1% and 2% doses.

Although use of the 0.5% dose of stinging nettle led to the highest number of lymphocytes, the level was the same as the controls. Also, monocytes and neutrophils numbers were high after use of the 1% dose of stinging nettle, compared with the other doses and the controls. Overall, use of 2% stinging nettle led to the highest count of thrombocytes.

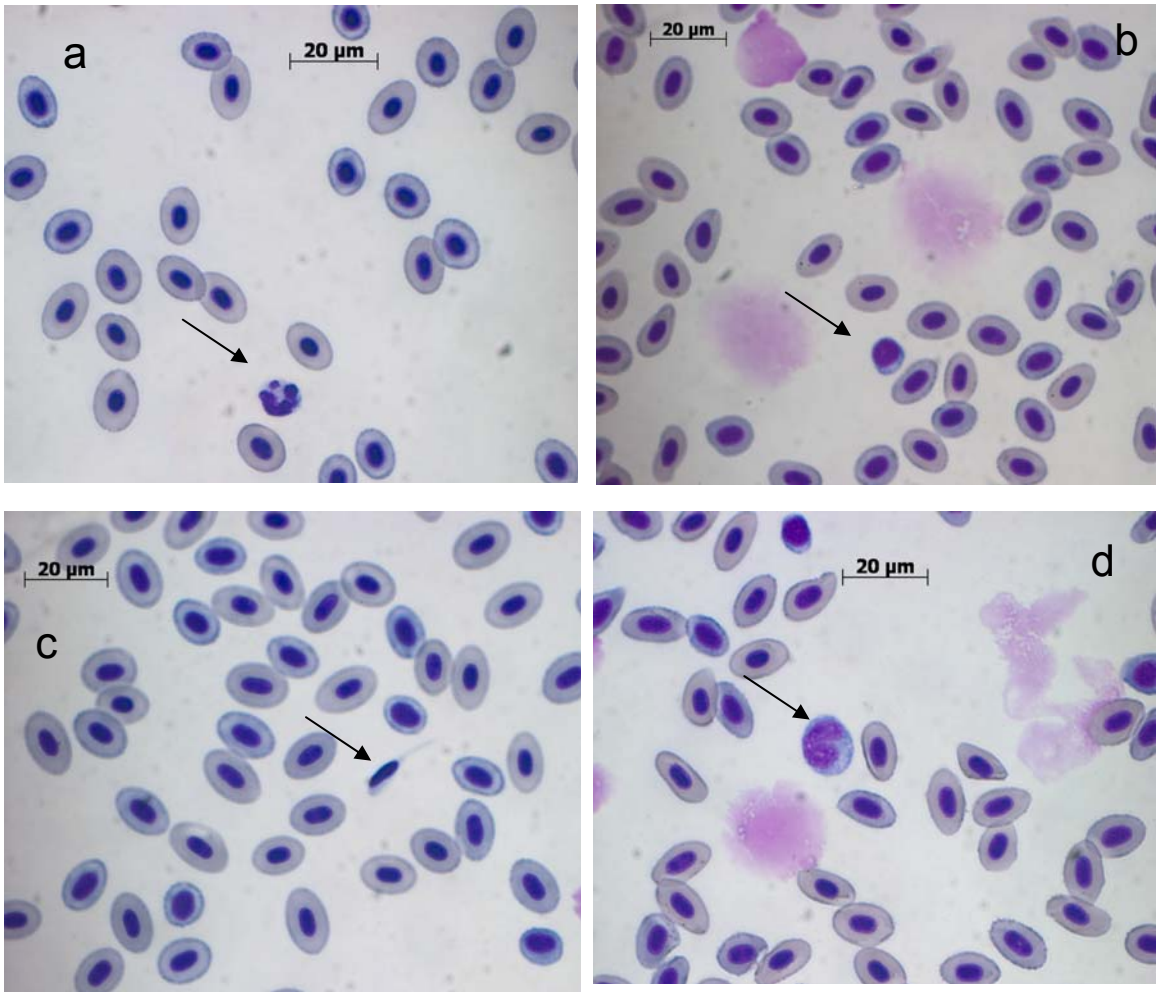


Fig. 3.4 Differential leucocytes and thrombocyte counts, a) neutrophils, b) lymphocyte, c) thrombocyte and d) monocytes

Table 3.4 Leukocyte and thrombocyte counts in rainbow trout fed for 14 days with 0.5%, 1% and 2% of lupin, mango and stinging nettle.

	Dose	**Lymphocytes**	**Monocytes**	**Neutrophils**	**Thrombocytes**
Lupin	0.5%	89 ± 1.52	2 ± 0.01	2 ± 0.33	8 ± 1.20
	1%	77 ± 3.84	6 ± 1*	4 ± 1.15	13 ± 1.73*
	2%	85 ± 1.20	6 ± 0.88*	3 ± 0.57	6 ± 0.88
Mango	0.5%	87 ±1.20	2 ± 0.33	1 ± 0.33	10 ± 0.57*
	1%	82 ±1.5	7 ± 0.67*	4± 0.88	7 ± 2.52
	2%	87 ± 5.03	4 ± 1.67	3 ± 1.15	6 ± 2.33
Stinging nettle	0.5%	88 ± 0.57	2 ± 0.57	3 ± 0.57	7 ± 0.57
	1%	80 ± 4.67	7 ± 1.33*	5 ± 0.67	8 ± 1.45
	2%	82 ± 5.57	6 ± 0.88*	3 ± 2.67	9 ± 2.08
Control		88 ± 1.73	4 ± 0.57	3 ± 1.20	5 ± 0.33

Data represented as mean ± SE.

(*)= $P < 0.05$.

3.5 Induced humoral immune responses after feeding for 14 days with dietary supplements.

3.5.1 Lysozyme activity

Generally, lysozyme activity was highest in fish fed with 2% lupin (1100 unit/ml), mango (1016.67 unit/ml) ($p < 0.05$) and stinging nettle (1002.08 unit/ml), (Fig. 3.5). Levels were less in the groups which received 1% and 0.5% doses. The controls revealed the lowest value of 797.91 unit/ml. It should be emphasised that there were significant differences in data from fish fed with 1% and 2% mango compared to the controls ($p < 0.05$).

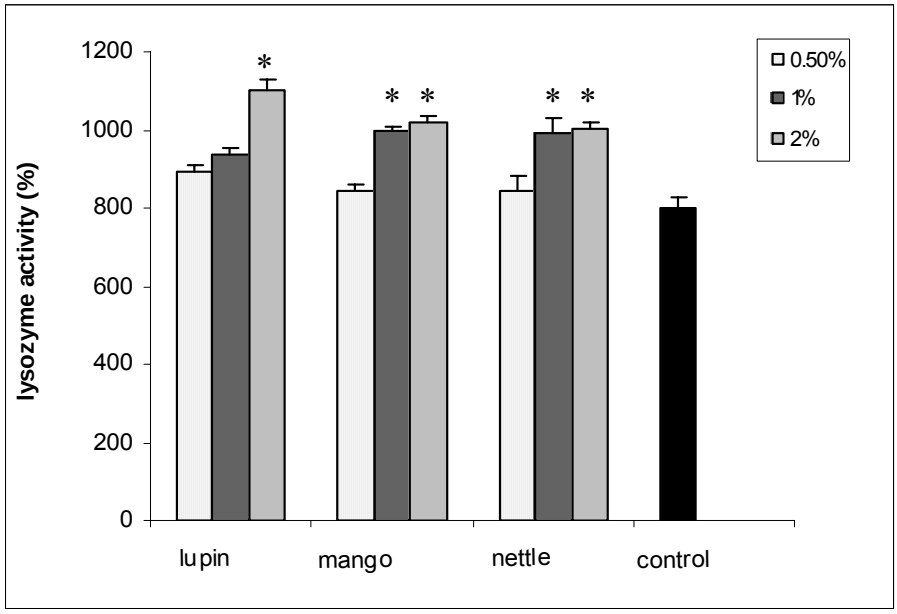


Fig. 3.5 Lysozyme activity of rainbow trout fed for 14 days with 0.5%, 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.5.2 Bactericidal activity

With mango, lupin and stinging nettle, the lowest number of colonies developing on TSA occurred with the 1% dose (82 x 10^3, 78 x 10^3 and 56 x 10^3, respectively) with highly significant differences to the control, 194 x 10^3 ($p < 0.05$). This level of activity was followed in turn by the 2% and 0.5 % doses (Fig. 3.6).

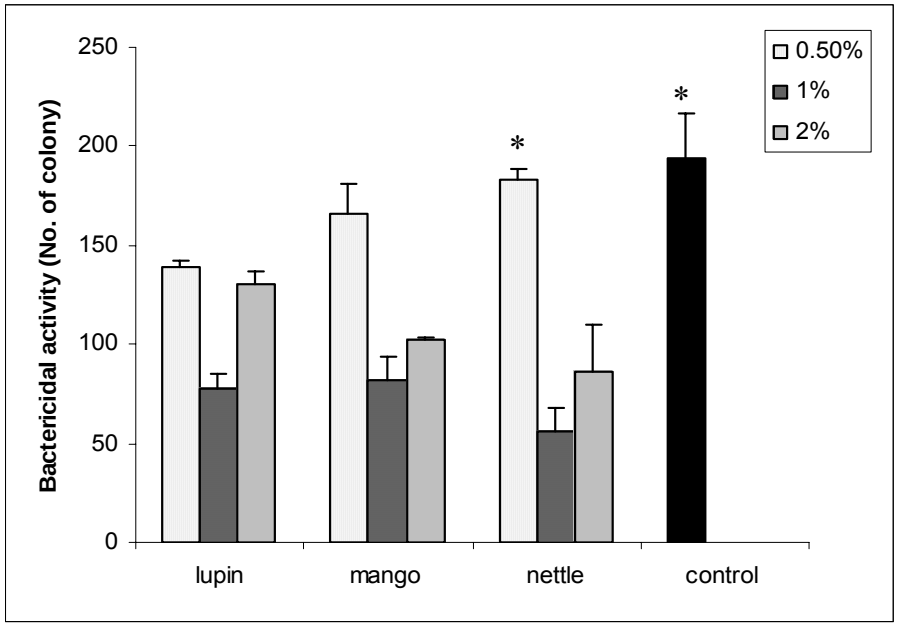


Fig. 3.6 Bactericidal activity of rainbow trout fed for 14 days with 0.5%, 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.5.3 Antiprotease content

The serum anti-trypsin activity was higher in fish fed with the 2% dose of lupin ($p < 0.05$), (50.28 %) followed by the 0.5% (49.88%) and 1% dose (49.03%), (Fig. 3.7). In contrast, the highest activity occurred with the 1% doses of both mango and stinging nettle ($p < 0.05$) (50.49% and 49.03%, respectively) followed by the 2% and 0.5% doses. Generally, the control showed the lowest activity, i.e. 41.8 %.

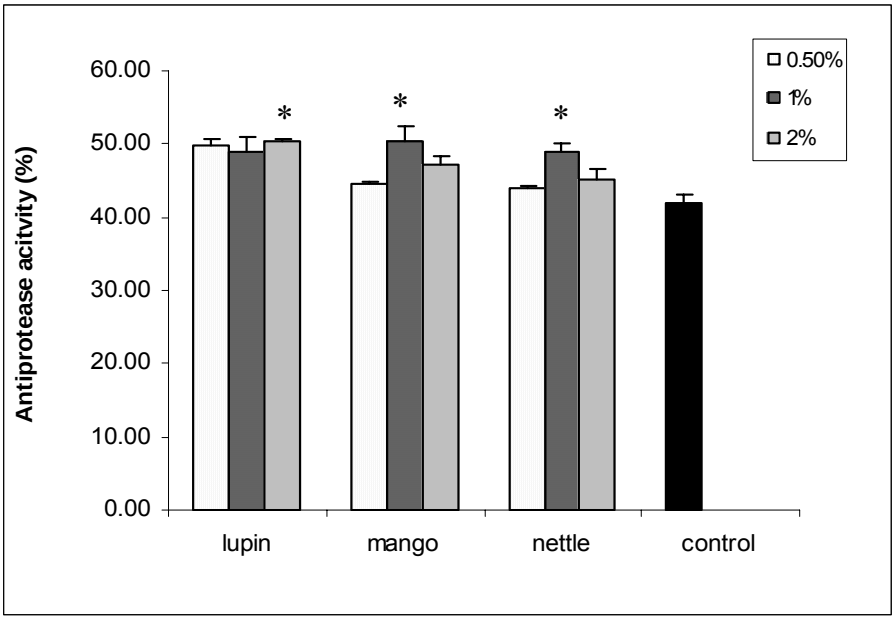


Fig. 3.7 Antiprotease activity of rainbow trout fed for 14 days with 0.5%, 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.5.4 Alternative complement activity

The use of 2% doses of lupin, mango, and stinging nettle led to the highest level of complement activity (47.96 unit/ ml, 55.74 unit/ ml and 51.36 unit/ ml, respectively) ($p < 0.05$), (Fig. 3.8), followed by the 1% and 0.5% doses. Generally, the controls revealed the lowest value (6.62 unit/ ml) as compared with the treatment groups. Moreover, doses in mango recorded the highest result as compared with lupin and stinging nettle.

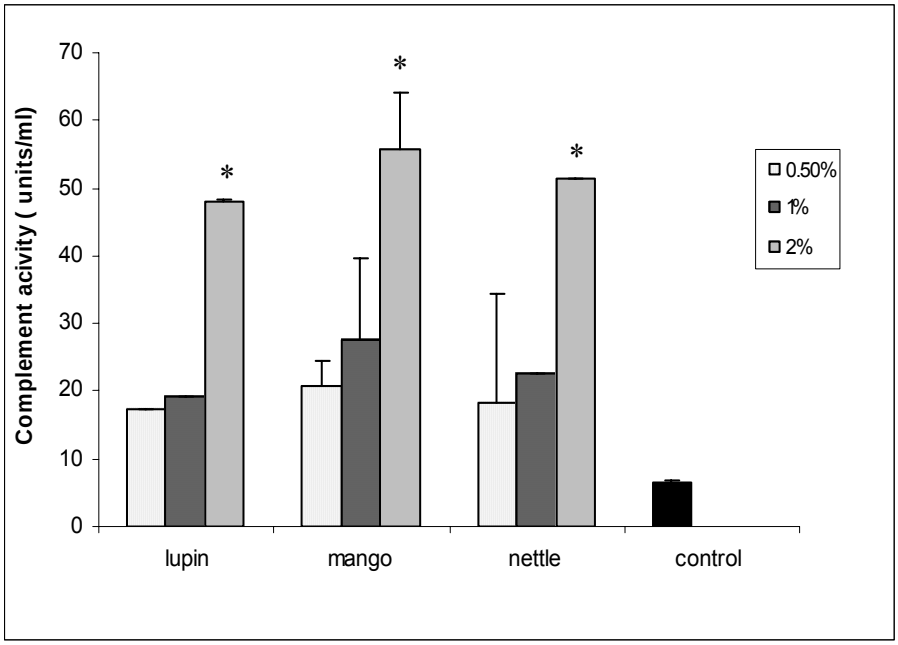


Fig. 3.8 Alternative complement activity of rainbow trout fed for 14 days with 0.5%, 1% and 2% of lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.5.5 Myeloperoxidase content

The highest value in myeloperoxidase (Fig. 3.9) was recorded in the 2% and 1% doses of all the treatment groups (with a significant difference to the controls, $p < 0.05$). This was followed by the level achieved with the 0.5 % doses. The controls revealed the lowest value as compared to the treatment groups (OD = 0.24).

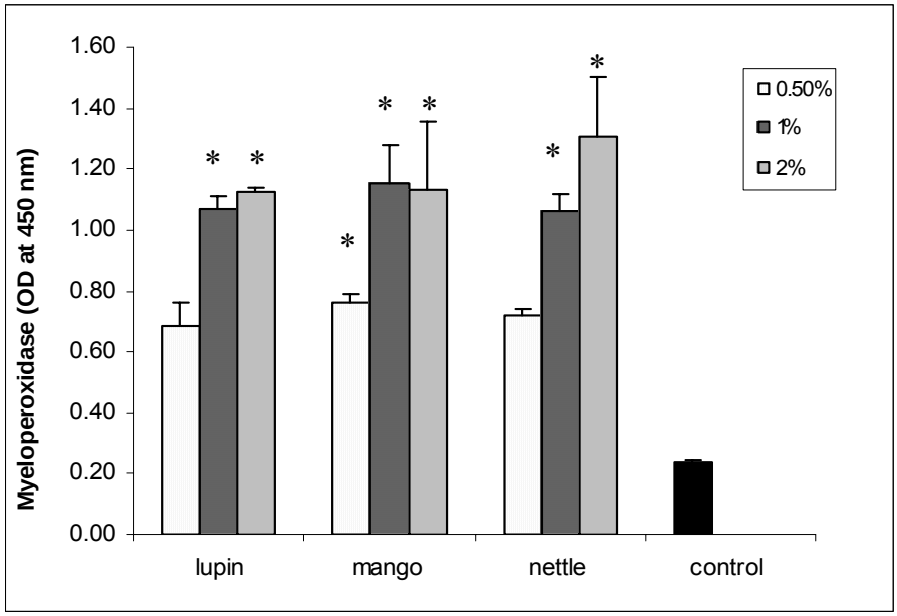


Fig. 3.9 Myeloperoxidase content of rainbow trout fed for 14 days with 0.5%, 1% and 2% of lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.5.6 Total protein

The 1% dose of lupin led to the highest protein value (7.78 g/dl) in recipient fish, with the level followed by 2% dose (6.74 g/dl) (significant difference with the controls, $p < 0.05$), and the 0.5% dose (5.59 g/dl), respectively (Fig. 3.10). Doses of 1% and 2% mango (8.59 and 7.01) g/dl led to levels which were also significantly higher than the controls (2.45 g/dl), ($p < 0.05$). Also, with 1% stinging nettle the value of 6.49 g/dl was recorded, which is significant compared with the controls ($p < 0.05$) The values achieved with 2% and 0.5% stinging nettle were 6.2 and 5.87 g/dl, respectively.

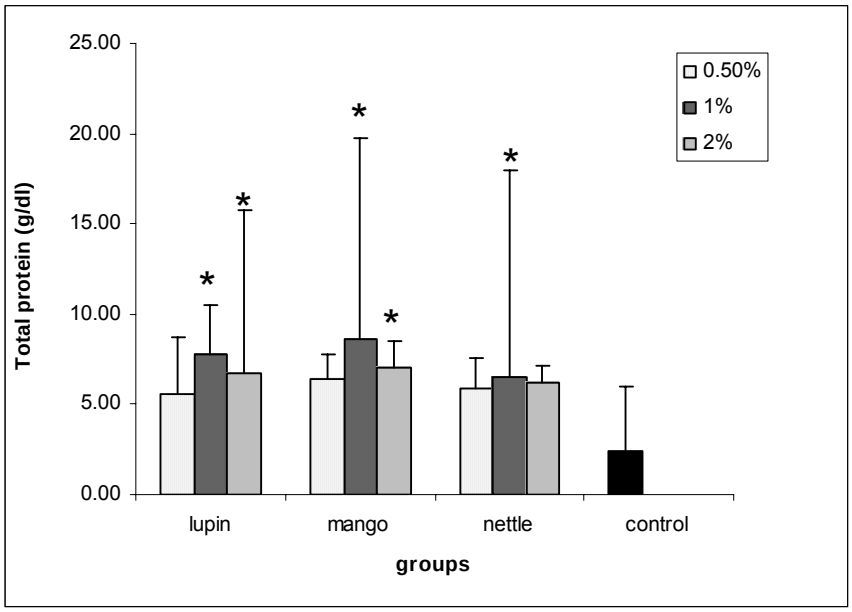


Fig. 3.10 Total protein levels in rainbow trout fed for 14 days with 0.5%, 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.5.7 Albumin

The highest value was recorded in fish fed with 0.5% lupin (0.47 g/dl), (significant difference with the controls, $p < 0.05$), followed by the 2% (0.42 g/dl) and 1% doses (0.39 g/dl), respectively (Fig. 3.11). With mango, the highest value was achieved with the 1% dose (0.43 g/dl), whereas the results for the 0.5% and 2% dose were the same, i.e. 0.4 g/dl.

The 0.5% dose of stinging nettle led to 0.49 g albumin /dl, which was the highest level for this dietary supplement ($p < 0.05$), and was followed by the 1% (0.43 g/dl) and 2% doses (0.41 g/dl), respectively.

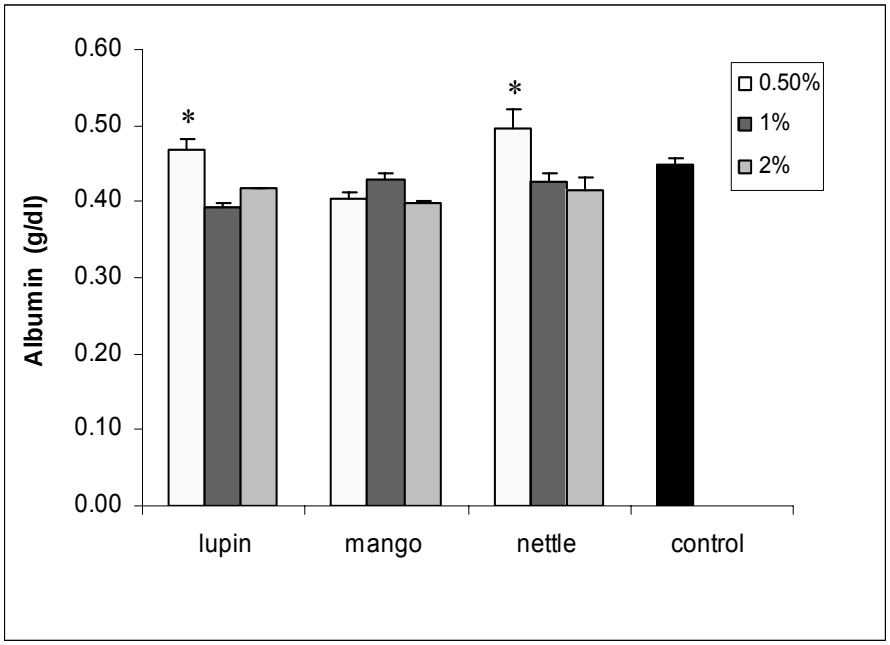


Fig. 3.11 Albumin levels of rainbow trout fed for 14 days with 0.5%, 1% and 2% of lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.5.8 Globulin

The highest value for globulin resulted from use of 1% doses of lupin, mango and stinging nettle ($p < 0.05$), followed by the 2% and 0.5% doses, and the controls (Fig. 3.12). Generally all doses of mango led to higher globulin levels as compared with the same doses of lupin and stinging nettle.

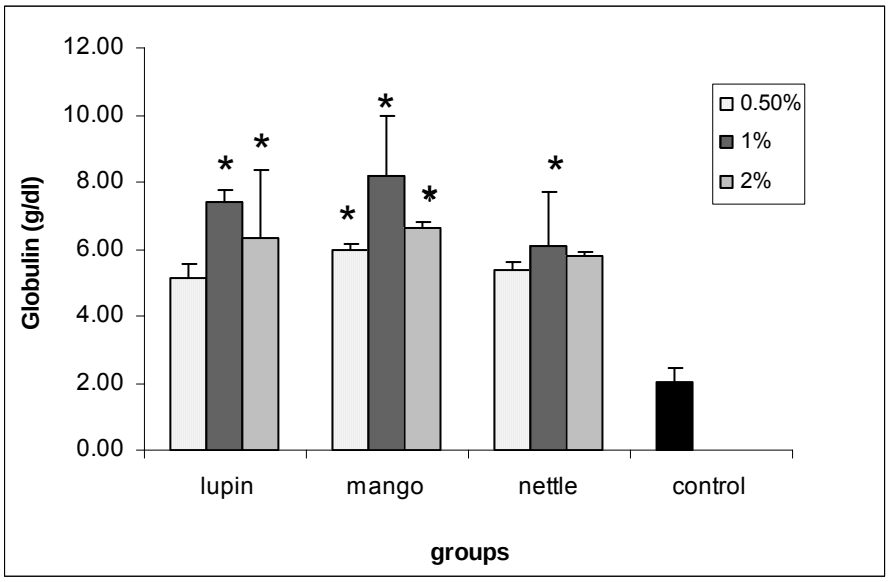


Fig. 3.12 Globulin levels of rainbow trout fed for 14 days with 0.5%, 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.6 Determination of the effective period of feeding with the dietary supplements to induce resistance to *A. hydrophila*

Experiments sought to compare the relative effects of feeding for 7, 14, 21 and 28 days using 1% lupin, mango and stinging nettle (Fig. 3.13). Here, the results demonstrated a significant ($p < 0.05$) increase in resistance to challenge with *A. hydrophila* following feeding for 14 days. In particular, after feeding for 7 days, lupin and stinging nettle led to higher percent survival, i.e. 80% ($p < 0.05$). Use of stinging nettle led to higher survival, i.e. 85%, after 21 days ($p < 0.05$), followed by mango (75%) and lupin (60%). In contrast, use of mango led to the highest survival after feeding for 28 days (75%), followed by stinging nettle (65%). Generally, with the exception of lupin (fed for 28 days) and mango (fed for 7 days), all the treatment groups led to higher survival than the controls.

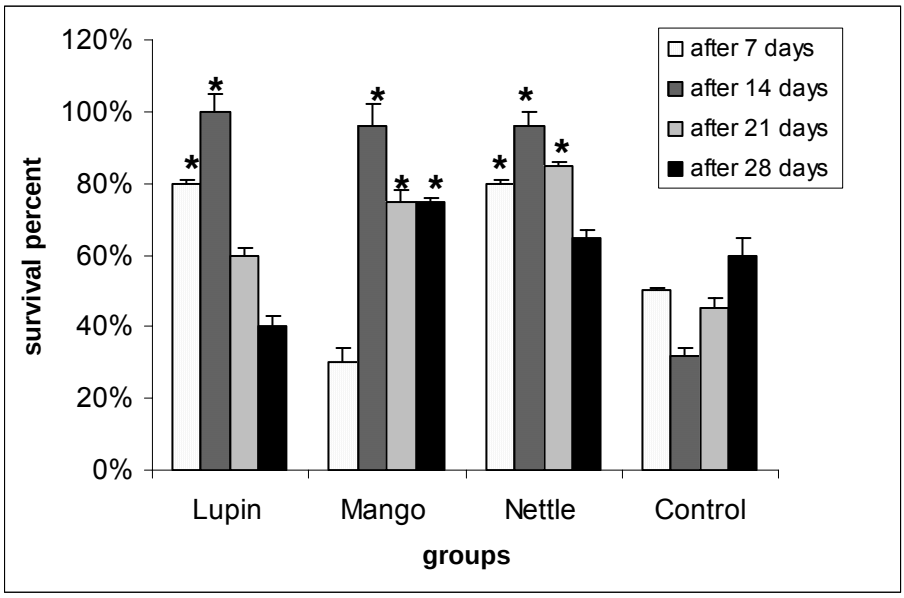


Fig. 3.13 Survival of rainbow trout fed for 7, 14, 21 and 28 days with 1% lupin, mango and stinging nettle followed by challenge with *A. hydrophila* (data for 14 day is obtained from fig. 3.1)

(*) = significant difference with control at the same day ($p < 0.05$)

Bars = mean ± S.E.

Feeding fish with 2 % of lupin, mango or stinging nettle for 7, 14, 21 and 28 days led to different levels of resistance to challenge with *A. hydrophila* (Fig. 3.14). Feeding for 21 days with 2% mango or stinging nettle ($p < 0.05$) (78% and 88% survival, respectively) led to the highest survival after injection with *A. hydrophila*. This level was followed by the results for the 14 day feeding regime. The highest level of survival in fish fed with 2% lupin was for the 14 day feeding regime (70% survival) followed by 21 days (64% survival). Moreover, feeding for 7 with 2% of any of the three plants did not reveal any substantial difference except in the case of stinging nettle, which led to a higher survival (64%) compared with the control (28%).

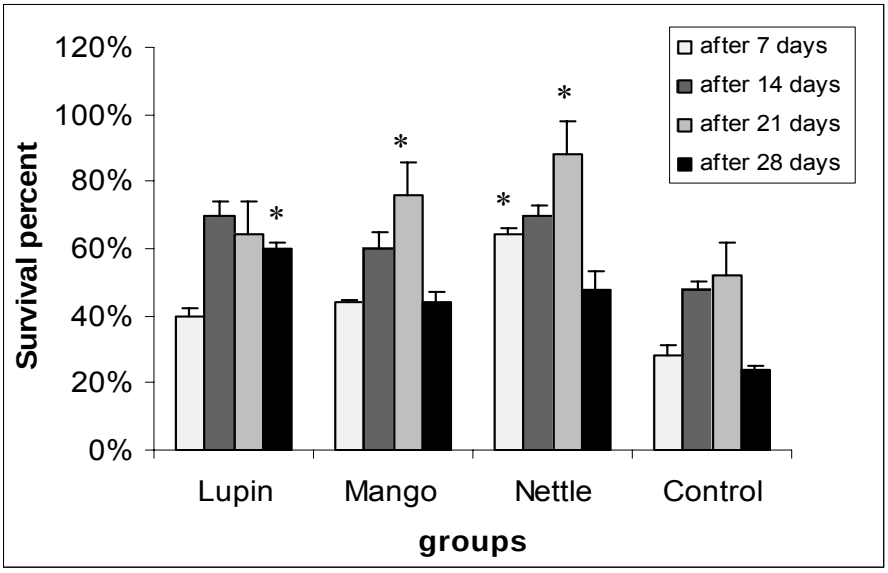


Fig. 3.14 Survival of rainbow trout fed for 7, 14, 21 and 28 days with 2 % lupin, mango and stinging nettle followed by challenge with *A. hydrophila* (data for 14 day is obtained from fig. 3.1)

(*) = significant difference with control at the same day ($p < 0.05$)

Bars = mean ± S.E.

3.7 Determination of the effective period of feeding with dietary supplements to induce humoral immune responses

The 1% and 2% doses of lupin, mango and stinging nettle led to much more pronounced effects than the 0.5% doses. Therefore, emphasis was placed on feeding fish with 1% and 2% doses of the three dietary supplements.

3.7.1 Lysozyme activity

The highest significant lysozyme activity was recorded after feeding for 14 days with 1% mango and stinging nettle (1000 and 993.75 units/ml, respectively) ($p < 0.05$) followed by 21 and 28 day feeding regimes, respectively (Fig. 3.15). Also, feeding for 14 days with 1% lupin showed a higher and significant activity (935.42 units/ml) ($p < 0.05$), followed by 28 and 21 day feeding regimes. Generally, feeding for 7 days with 1% lupin, mango and stinging nettle resulted in the lowest activity. The highest significant lysozyme activity ($p < 0.05$) was recorded after feeding for 14 days with 2% lupin, mango and stinging nettle (1100, 1016.67 and 1002.08 units/ml, respectively) (Fig. 3.15). With the exception of lupin after 28 days, there was a decline in lysozyme activity at 21 and 28 days. The lowest activity was recorded after 7 days ($p < 0.05$).

Also, feeding for 7 days recorded the lowest lysozyme activity in the controls (402.08 units/ml) ($p < 0.05$). Moreover, the highest activity in the controls was recorded after 14 days (797.92 units/ml), followed by a gradual decline in activity at 21 and 28 days. Lysozyme activity of lupin was higher than the corresponding days of the controls, except after feeding with 2% doses for 21 and 7 days. With mango, the activity was higher than the controls only at 14 days, and after feeding for 7 days with the 1% dose. The use of 1% stinging nettle led to a higher activity than the corresponding control, but only feeding for 14 days with the 2% dose revealed a higher activity more than its control ($p < 0.05$).

3.7.2 Alternative complement activity

Feeding for 7 days with 1% lupin and mango revealed the highest complement activity ($p < 0.05$) (32.01 and 29.33 units/ml), followed by 21, 14, and 28 days feeding regimes respectively. With 1% stinging nettle, 21 days of feeding led to the highest value (32.09 units/ml), followed by 7, 14 and 28 day feeding regimes (Fig. 3.16). Feeding for 14 days with 2% lupin, mango and stinging nettle led to the highest significant values ($p < 0.05$) (47.96, 55.74 and 51.36 units/ml, respectively), followed by 7, 21 and 28 day feeding regimes (Fig. 3.16). In contrast, the highest value in the control was at 7 days (31.54 units/ml) followed by 21, 28 and 14 days. From a comparison between each treatment and the corresponding control at the same day, it was apparent that both 1% and 2% lupin were higher than the controls, except after 21 days in the case of 1% lupin. This was especially the case after 14 and 28 days when there was a highly significant difference compared with the controls ($p < 0.05$). With the exception of a 7 day feeding regime, both 1% and 2% mango led to high values compared to the corresponding control. Similarly, both doses of stinging nettle, showed higher values compare with the controls, except in the case of feeding with 2% stinging nettle for 7 days.

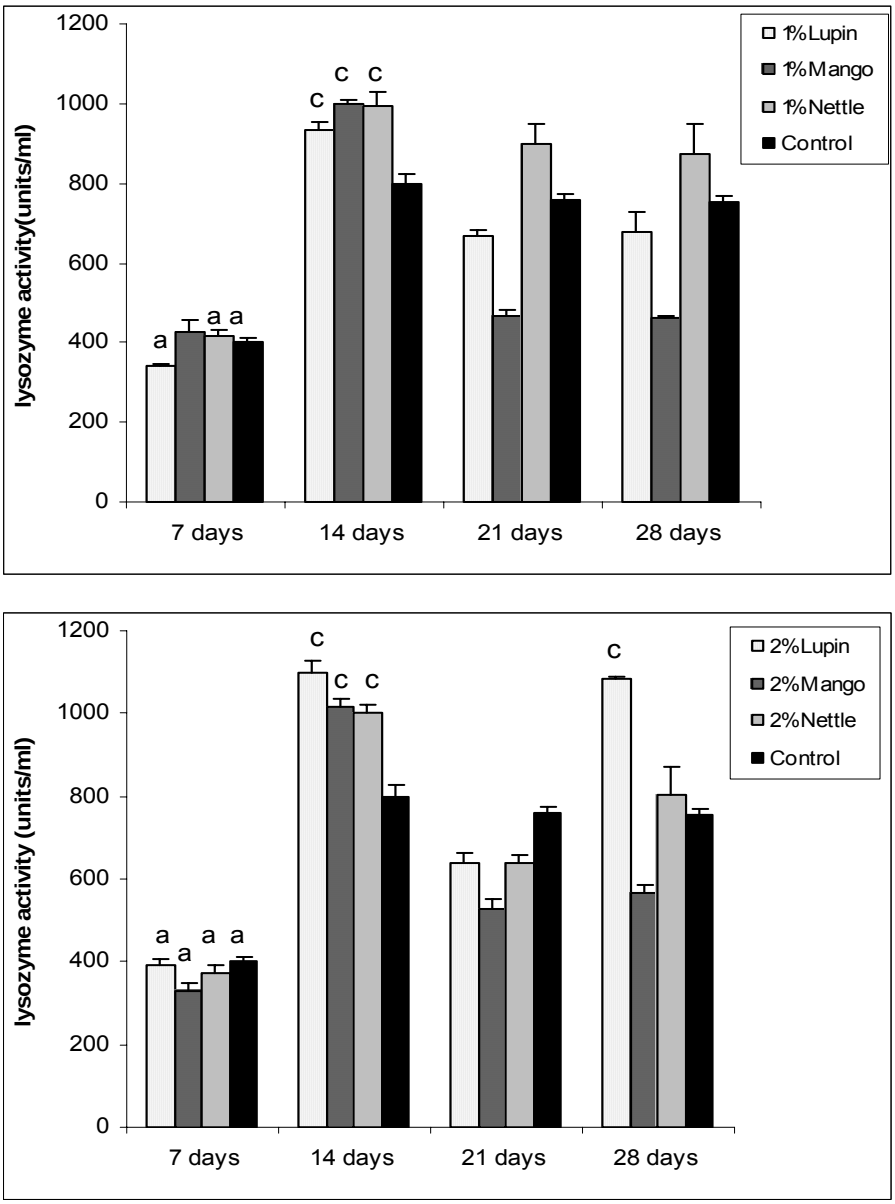


Fig. 3.15 Lysozyme activity of rainbow trout fed for 7, 14, 21 and 28 days with 1% and 2% of lupin, mango and stinging nettle (data for 14 day is obtained from fig. 3.5)

Bars = mean ± S.E.

a = Significant difference between treatment at 7, 14, 21 and 28 days ($p < 0.05$)

b = Significant difference between treatment and controls on the same day ($p < 0.05$)

c = Significant difference between treatment at 7, 14, 21 and 28 days and with the control on the same day ($p < 0.05$)

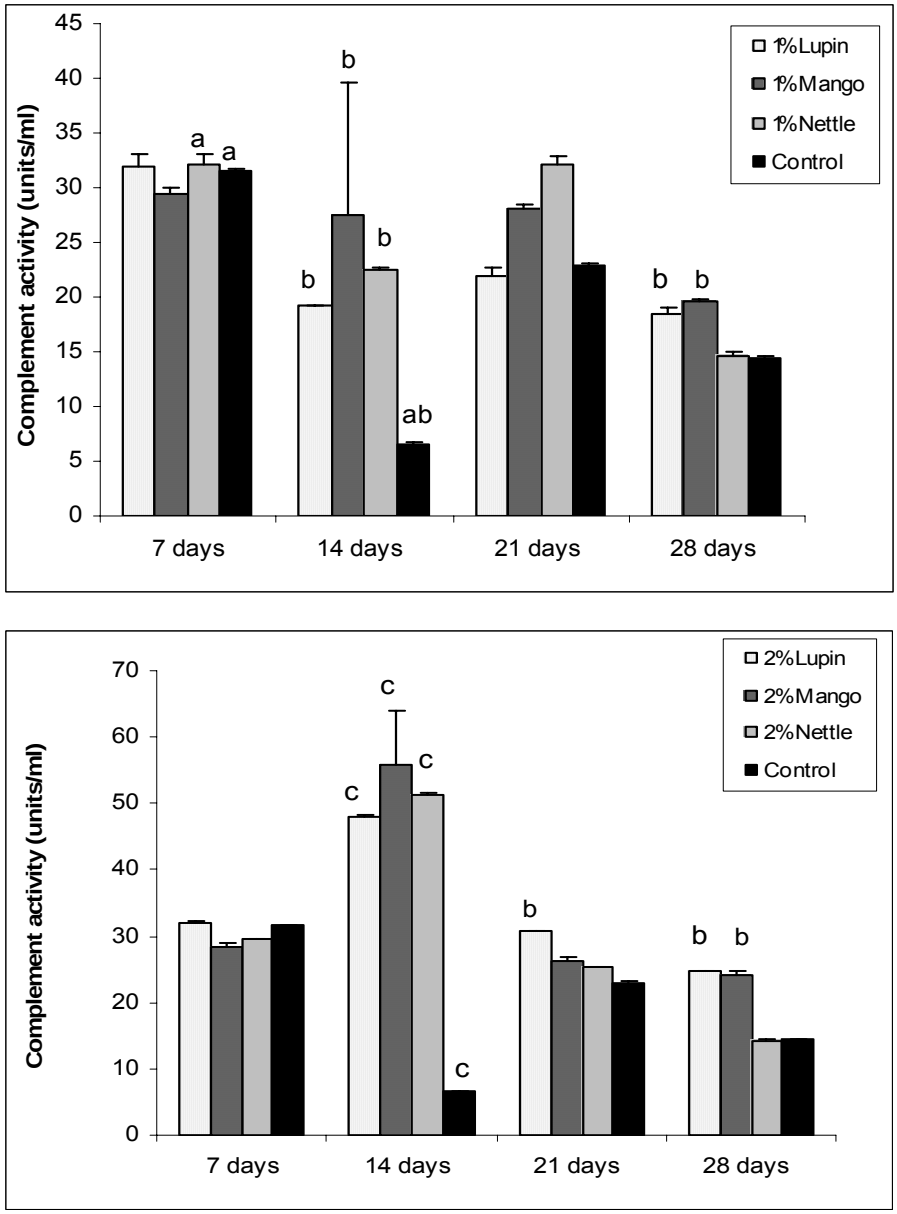


Fig. 3.16 Alternative complement activity of rainbow trout fed for 7, 14, 21 and 28 days with 1% and 2% lupin, mango and stinging nettle (data for 14 day is obtained from fig. 3.8)

Bars = mean ± S.E.

a = Significant difference between treatment at 7, 14, 21 and 28 days ($p < 0.05$)

b = Significant difference between treatment and controls on the same day ($p < 0.05$)

c = Significant difference between treatment at 7, 14, 21 and 28 days and with the control on the same day ($p < 0.05$)

3.7.3 Myeloperoxidase content

Feeding for 7 days with 1% of lupin, mango and stinging nettle (1.46, 1.45, and 1.12 OD, respectively) resulted in the highest significant myeloperoxidase content ($p < 0.05$). However, the levels decreased gradually at 14 and 21 days, although still significant, till 28 days ($p < 0.05$) (Fig. 3.17). Feeding for 7 and 14 days with 2% lupin and stinging nettle led the highest significant myeloperoxidase value ($p < 0.05$) (Fig. 3.17) as compared with 21 and 28 day feeding regimes when there was a gradual decrease in value. Interestingly, the highest significant quantity in feed with 2% mango was recorded after 7, 14 and 21 days (1.18, 1.13 and 1.21 OD, respectively) as compared with 28 days (OD = 0.24 nm). The highest significant value in the controls was recorded after 7 days (OD = 1.18), although feeding for 14, 21 and 28 days recorded the lowest actual levels (0.24, 0.49 and 0.25 OD, respectively). All doses of lupin led to higher myeloperoxidase content compared with the controls especially after feeding with 1% amounts for 14 and 21 days and after feeding with 2% for 14 days, with the data showing a significant difference to the controls ($p < 0.05$). Similarly, all the durations and doses feeding with supplemented diets with mango revealed higher values compared with the corresponding controls, except for the 2% doses which were fed for after 28 days ($p < 0.05$). Also, feeding for 14 days with stinging nettle revealed highly significant values as compared with the controls. Similarly feeding for 7 and 21 days with 1% and 2% stinging nettle, respectively, resulted in higher values compared with the controls.

3.7.4 Antiprotease activity

The highest significant antiprotease activity was recorded in fish which were administered with 1% lupin and 1% stinging nettle for 21 days (72.81% and 71.56%, respectively) ($p < 0.05$) followed in terms of activity by 28 days and 7 days feeding periods (Fig. 3.18). Also, the highest significant activity ($p < 0.05$) was recorded after feeding with 1% mango (73.96 %) for 21 day. Conversely, the lowest value was recorded in the group which was fed with 1% mango for 28 days (49.40%). The highest significant antiprotease activity achieved with 2% lupin, mango and stinging nettle ($p < 0.05$) was after feeding for 21 days (70.73%, 73.07%, and 73.07% respectively), followed by the 28 day feeding regime (Fig. 3.18). With the exception of stinging nettle, the lowest activity was recorded after feeding periods of 7 days. Also, the control

group at 21 days revealed the highest significant activity (70.26%) ($p < 0.05$) followed by data for 7 days (52.51%). Overall, all the treatment groups demonstrated higher antiprotease activity compared to the control except after 7 days; most of the differences being significant.

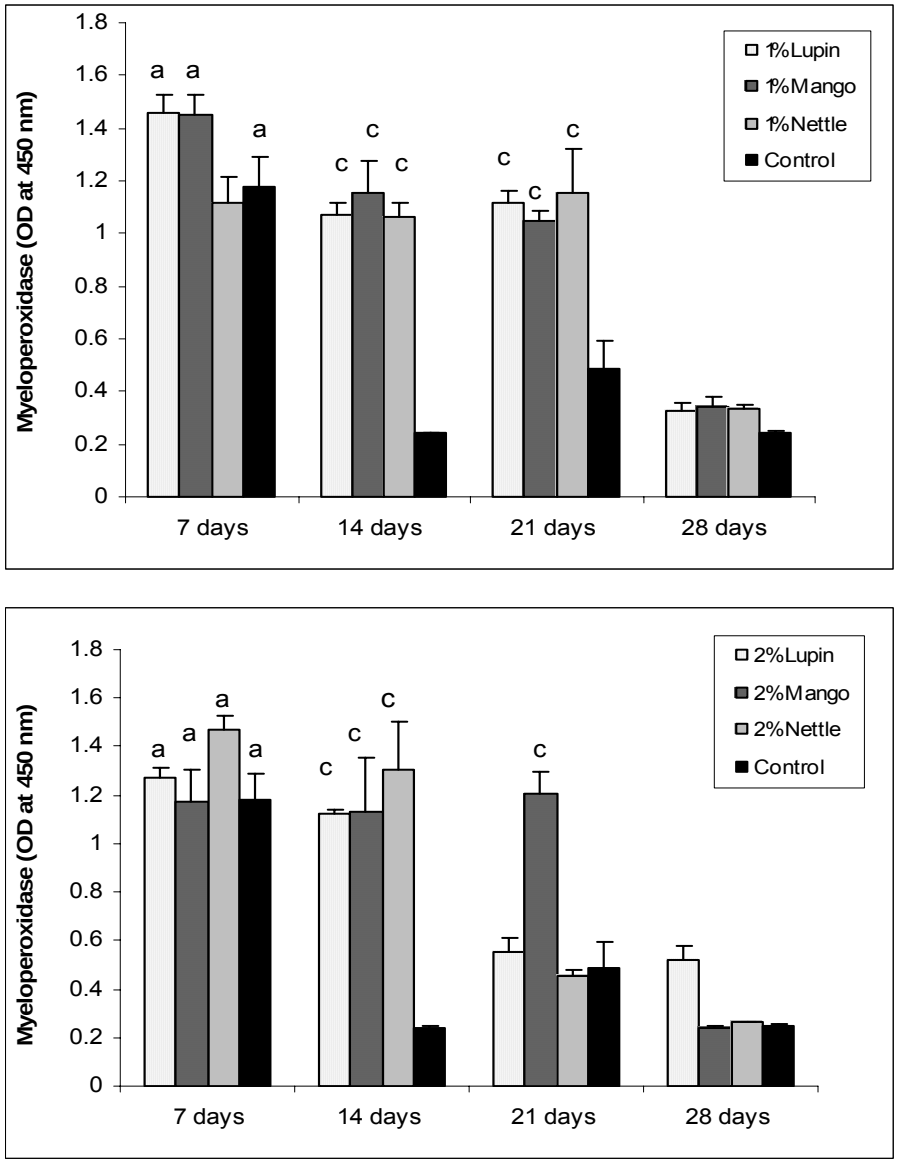


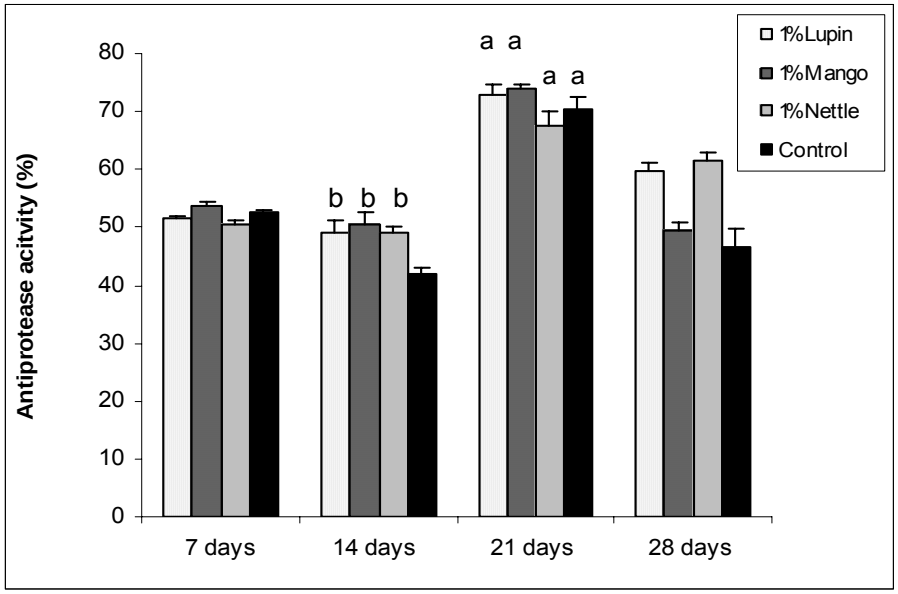
Fig. 3.17 Myeloperoxidase content of rainbow trout fed for 7, 14, 21 and 28 days with 1% and 2% lupin, mango and stinging nettle (data for 14 day is obtained from fig. 3.9)

Bars = mean ± S.E.

a = Significant difference between treatment at 7, 14, 21 and 28 days ($p < 0.05$)

b = Significant difference between treatment and controls on the same day ($p < 0.05$)

c = Significant difference between treatment at 7, 14, 21 and 28 days and with the control on the same day ($p < 0.05$)



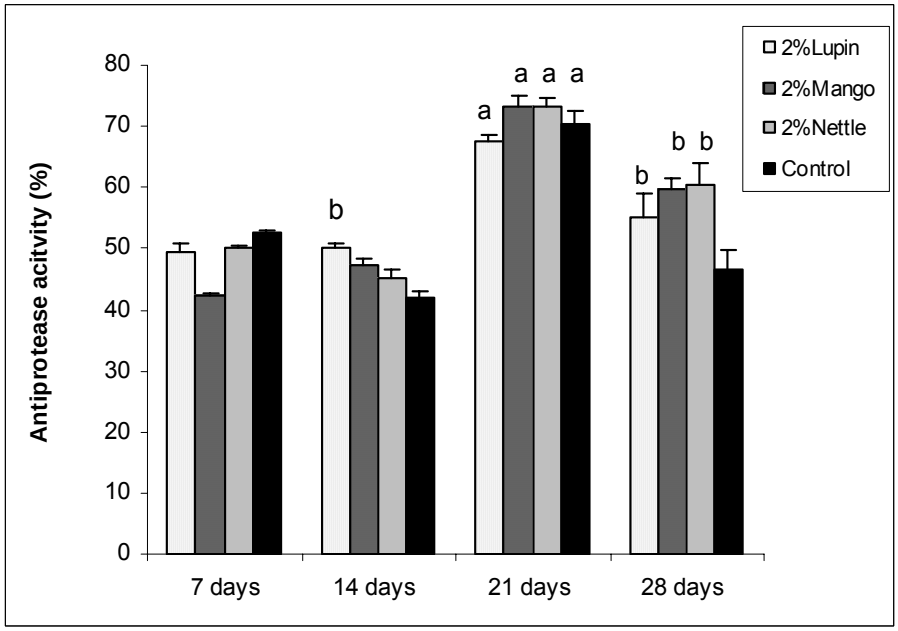


Fig. 3.18 Antiprotease activity of rainbow trout fed for 7, 14, 21 and 28 days with 1% and 2% lupin, mango and stinging nettle (data for 14 day is obtained from fig. 3.7)

Bars = mean ± S.E.

a = Significant difference between treatment at 7, 14, 21 and 28 days ($p < 0.05$)

b = Significant difference between treatment and controls on the same day ($p < 0.05$)

3.7.5 Bactericidal activity

The lowest bactericidal activity was after feeding for 7 days with 1% lupin and 1% stinging nettle (114 x 10^3 and 101 x 10^3, respectively) and after 14 days feeding with 1% mango (165 x 10^3); the activity reached maximum levels at 21 days (37 x 10^3, 58 x 10^3, and 44 x10^3, respectively) **(**Fig. 3.19**)**. Certainly, there was a highly significant difference between the highest and the lowest activity ($p < 0.05$). The lowest bactericidal activity was recorded after feeding for 7 days with 2% mango and 2% stinging nettle (94 x 10^3 and 100 x 10^3, respectively), with the activity increasing gradually to reach a maximum at 21 days (38 x 10^3 and 36 x 10^3 for mango and stinging nettle, respectively) (Fig. 3.19). Conversely, the highest activity in 2% lupin was after feeding for 28 days (56 x 10^3) and the lowest activity was recorded after feeding for 21 days. The highest activity in the controls was after 14 days (194 x 10^3) ($p < 0.05$). With exception of feeding for 21 days with 2% lupin and for 28 days with 2% mango, administration of both doses led to higher bactericidal activity compared to the control, especially stinging nettle after 14 and 21 days when a significant enhancement in the activity was recorded when compared to the corresponding controls control ($p < 0.05$).

3.7.6 Total protein

The highest protein value was recorded after 14 days of feeding with 1% lupin and 1% mango (7.78 and 8.59 g/dl, respectively) (Fig. 3.20), followed by the data for 7, 21 and 28 days. Moreover, administration for 14 days was highly significant ($p < 0.05$) when compared with data for 21 and 28 days. In contrast, feeding for 7 days (6.81 g/dl) in 1 % stinging nettle produced the highest value, which was followed by the results of the 14 days feeding period (6.49 g/dl), although there were not any significant differences between these results. Similarly, the highest protein values following administration of 2% lupin and 2% mango were recorded after 14 days (6.74 and 7.01 g/dl, respectively) (Fig. 3.20), followed by data for 7 days (6.60 and 6.40 g/dl, respectively), whereas the lowest values were after 21 and 28 days. Conversely, the highest protein value following use of 2% stinging nettle was in revealed at 7 days (6.73 g/dl), followed by 14 days (6.20 g/dl), albeit without any significant difference. In the controls, the highest protein value was after 7 days (5.62 g/dl; a significant result), and was followed by a decrease in protein. Generally, all the treatment groups led to higher protein values as

compared with the controls. This was certainly the case after 14 days when all the treatment groups were highly significant ($p < 0.05$) when compared with the controls.

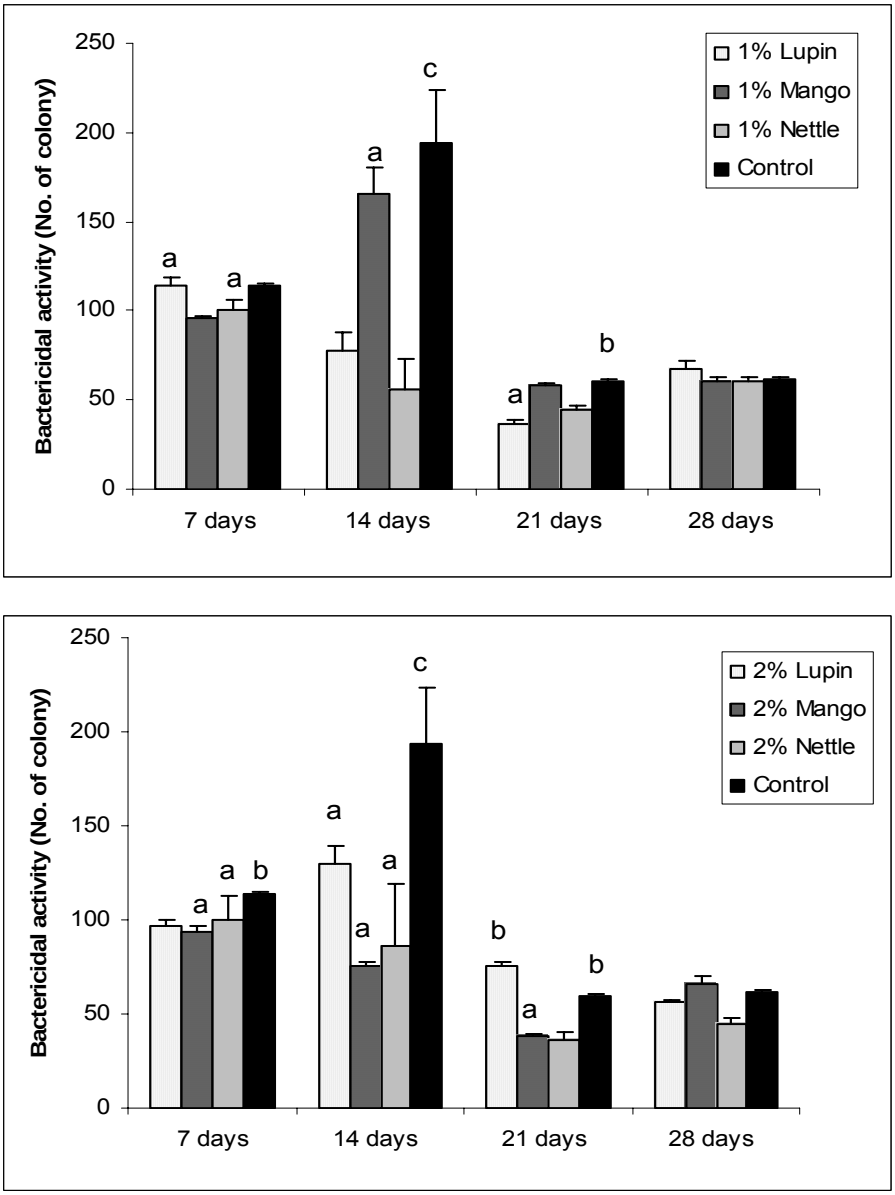


Fig. 3.19 Bactericidal activity of rainbow trout fed for 7, 14, 21 and 28 days with 1% and 2% lupin, mango and stinging nettle (data for 14 day is obtained from fig. 3.6)

Bars = mean ± S.E.

a = Significant difference between treatment at 7, 14, 21 and 28 days ($p < 0.05$)

b = Significant difference between treatment and controls on the same day ($p < 0.05$)

c = Significant difference between treatment at 7, 14, 21 and 28 days and with the control on the same day ($p < 0.05$)

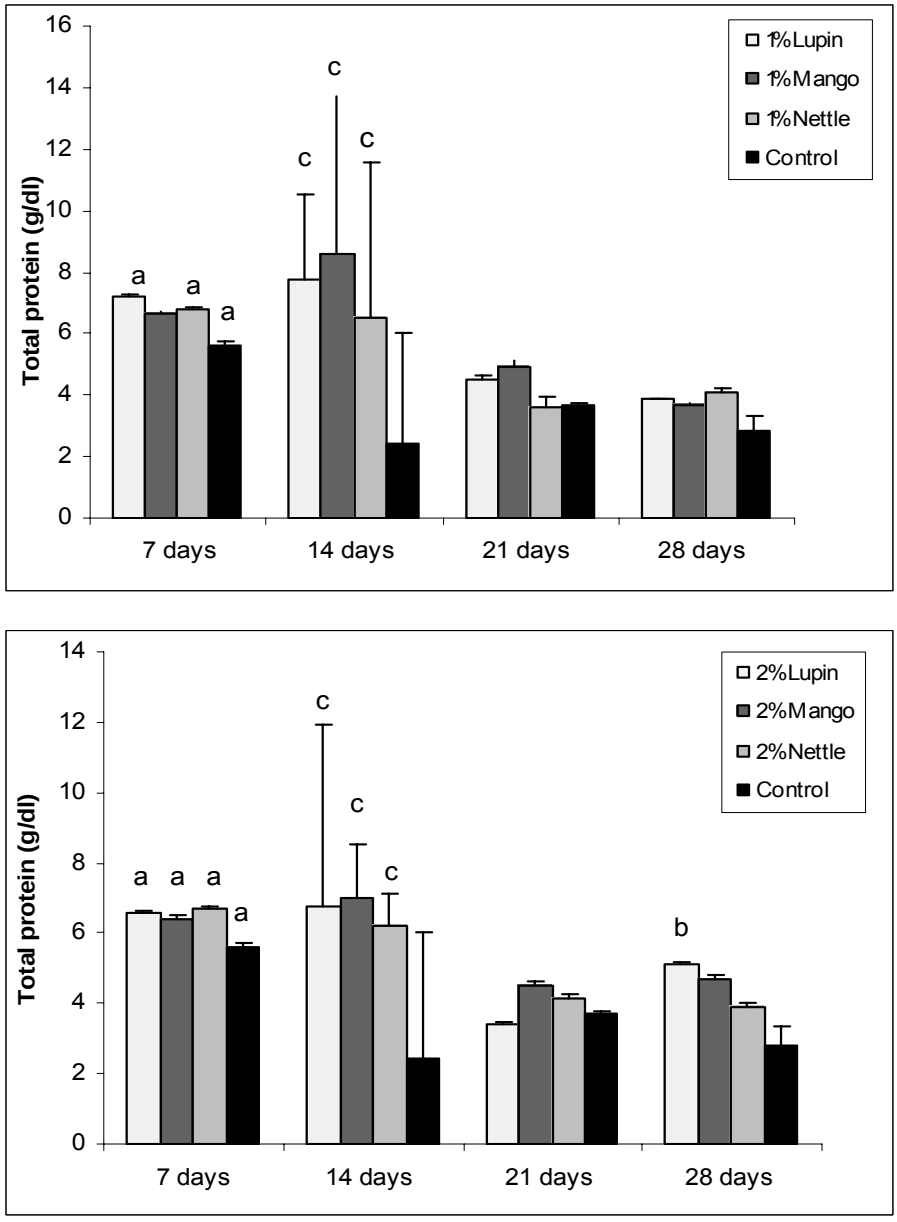


Fig. 3.20 Total protein of rainbow trout fed for 7, 14, 21 and 28 days with 1% and 2% lupin, mango and stinging nettle (data for 14 day is obtained from fig. 3.10)

Bars = mean ± S.E.

a = Significant difference between treatment at 7, 14, 21 and 28 days ($p < 0.05$)

b = Significant difference between treatment and controls on the same day ($p < 0.05$)

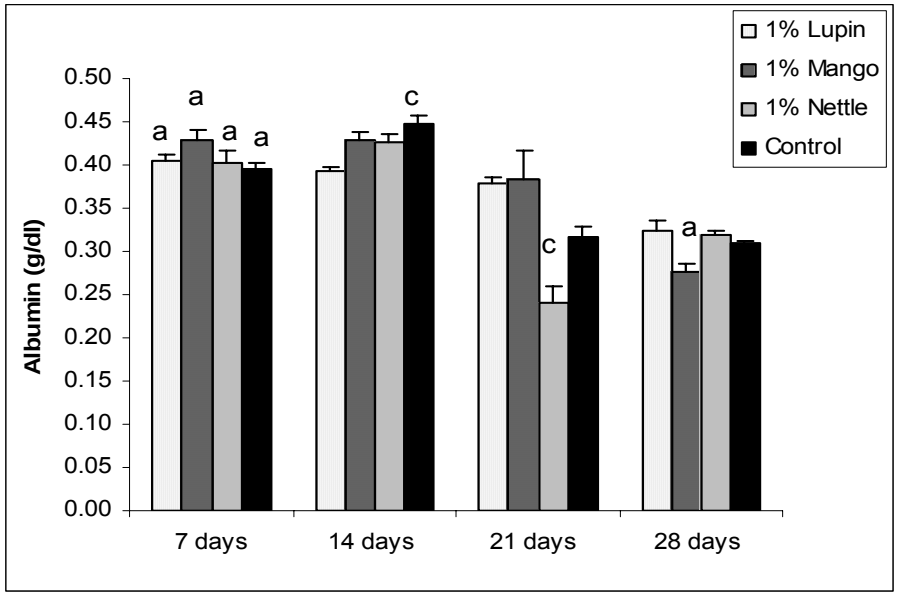
c = Significant difference between treatment at 7, 14, 21 and 28 days and with the control on the same day ($p < 0.05$)

3.7.7 Albumin

The highest albumin value was recorded in fish that had received 1% lupin, mango and stinging nettle fed over 7 and 14 days (Fig. 3.21). Then, the values declined to reach the lowest levels at 28 days in the case of lupin and mango (0.32 and 0.28 g/dl, respectively), and at 21 days with stinging nettle, (0.24 g/dl), (significant difference with the highest value, $p < 0.05$). Also, the highest albumin value was achieved after feeding for 7 and 14 days in 2% dose of lupin, mango and stinging nettle (Fig. 3.21). In contrast, the lowest values were recorded at 21 days with mango and stinging nettle (0.36 and 0.38 g/dl) and after 28 days with lupin (0.31 g/dl). Moreover, the highest albumin value in the controls was recorded after 14 days (0.45 g/dl), and demonstrated highly significant differences with the lowest value which was recorded at 28 days (0.31 g/dl). It was observed that the controls gave higher values then the treatment groups after 14 days, whereas they were virtually identical after 7 days. With the exception of 1% stinging nettle, feeding with the dietary supplements for 21 days led to higher values than the control. It was determined that 2% mango and 2% stinging nettle led to higher significant values after 28 days compared with their respective controls ($p < 0.05$).

3.7.8 Globulin

The highest globulin value was recorded after administration of 1% lupin and 1% mango for 14 days (7.39 and 8.16 g/dl) ($p < 0.05$). The values were less at 7, 21 and 28 days (Fig. 3.22). Conversely, use of 1% stinging nettle led to the highest values at 7 days (6.41 g/dl) followed by 14 days (6.07 g/dl). In contrast, the lowest values were recorded at 21 and 28 days (3.39 and 3.79 g/dl, respectively). Feeding for 14 days led to the highest globulin levels in the case of 2% lupin and 2% mango (6.32 and 6.61 g/dl, respectively) ($p < 0.05$), followed by results for 7 days (6.20 and 5.97g/dl, respectively). The lowest values after feeding 2% lupin and 2% mango were recorded after 21 and 28 days. Conversely, the highest value of 2% stinging nettle was recorded after feeding for 7 days (6.32 g/dl) ($p < 0.05$), followed by a gradual decrease to 28 days. All the treatment groups gave higher globulin values to their corresponding controls.



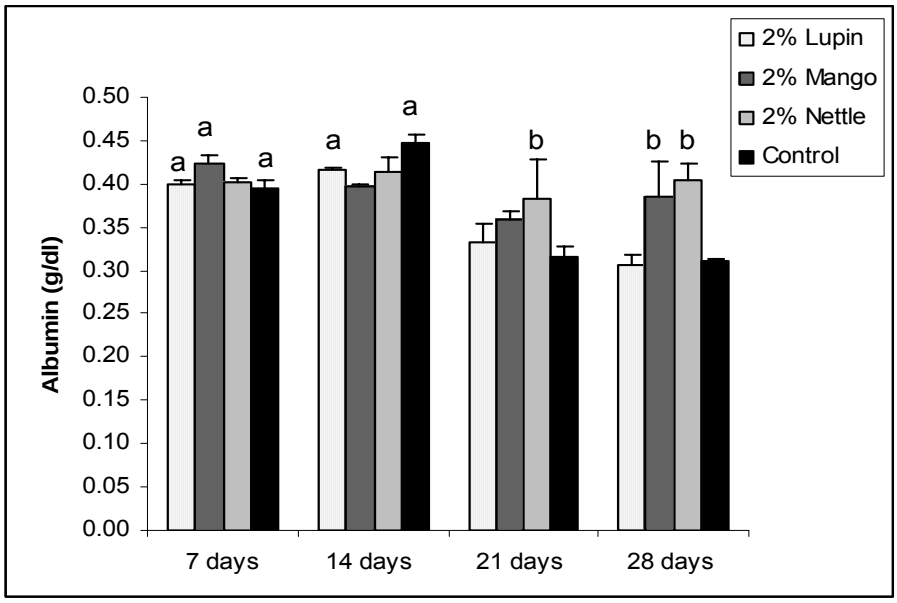


Fig. 3.21 Albumin levels in rainbow trout fed for 7, 14, 21 and 28 days with 1% and 2% lupin, mango and stinging nettle (data for 14 day is obtained from fig. 3.11)

Bars = mean ± S.E.

a = Significant difference between treatment at 7, 14, 21 and 28 days ($p < 0.05$)

b = Significant difference between treatment and controls on the same day ($p < 0.05$)

c = Significant difference between treatment at 7, 14, 21 and 28 days and with the control on the same day ($p < 0.05$)

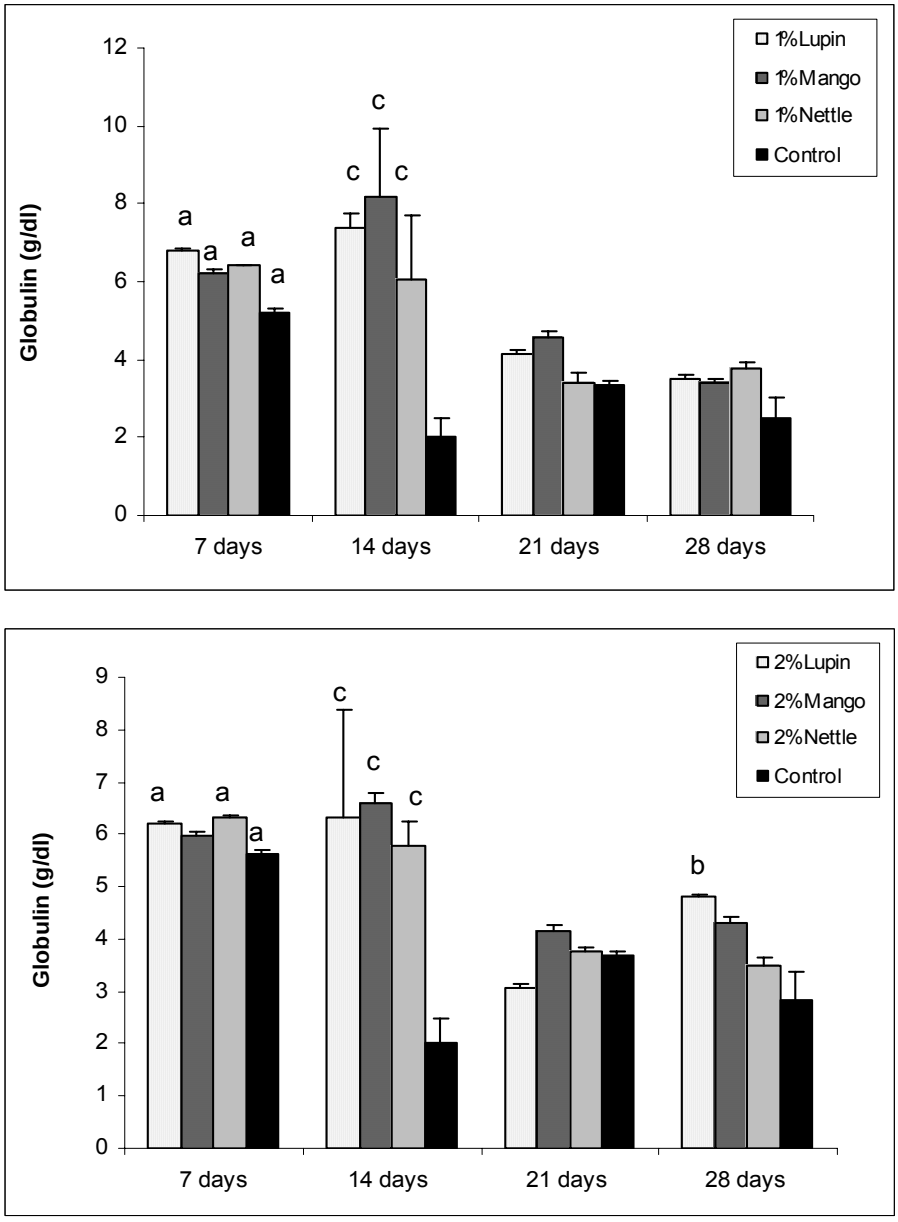


Fig. 3.22 Albumin levels of rainbow trout fed for 7, 14, 21 and 28 days with 1% and 2% lupin, mango and stinging nettle (data for 14 day is obtained from fig. 3.12)

Bars = mean ± S.E.

a = Significant difference between treatment at 7, 14, 21 and 28 days ($p < 0.05$)

b = Significant difference between treatment and controls on the same day ($p < 0.05$)

c = Significant difference between treatment at 7, 14, 21 and 28 days and with the control on the same day ($p < 0.05$)

3.8 Effect of long term feeding with dietary supplements on digestive enzymes

3.8.1 Total proteases in the stomach

Total proteases in the stomach which were examined at pH 1.5 in fish fed for 2 months with 2% lupin and stinging nettle, were 1.24 and 0.77 U/mg protein, respectively. The data indicated higher activity than the 1% doses (Fig. 3.23). Conversely, at the same pH, protease activity was higher in fish administered with 1% mango (1.05 U/mg protein) than the 2% dose (0.31 U/mg protein). Interestingly, the highest protease levels were recorded after administering 2% lupin (1.24 ± 0.59 U/mg protein) and 1% mango (1.05 ± 0.43 U/mg protein), and were significantly different to the controls (0.18 ± 0.01 U/mg protein) ($p < 0.05$). At pH 3.0, the total proteases in fish fed with 2% lupin, mango and stinging nettle (0.12, 0.1 and 0.11 U/mg protein, respectively) were higher than achieved with the 1% dose. Controls revealed the lowest value (0.004 ± 0.001 U/mg protein) which was significantly different to the 2% dose ($p < 0.05$). Generally, pH 1.5 led to higher values in total proteases than pH 3.0.

3.8.2 Total proteases in the intestine

Generally, total proteases (measured at pH 9.0) in the intestine of fish fed for 2 months were higher than data for pH 7.0 (Fig. 3.24). Moreover, at both pH 7.0 and 9.0, 2% lupin and mango led to higher protease levels than the 1% doses. Conversely, 1% stinging nettle led to higher protease activity in both pHs than the 2% dose. Although, the controls had the highest value (0.139 U/mg protein) at pH 7.0 but there was not ant significant difference between any of treatment groups and the controls. Also at pH 9.0, the controls did not reveal a significant difference with the treatment groups.

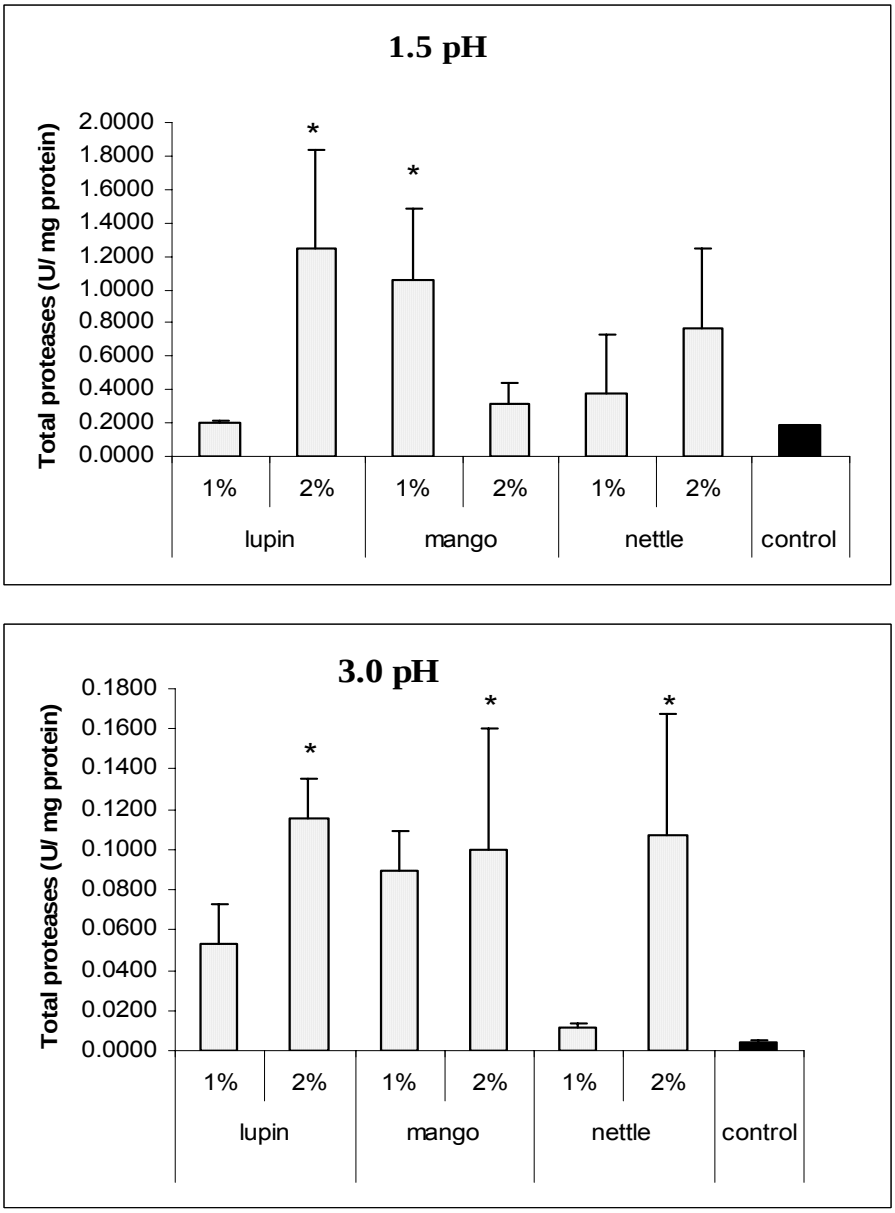


Fig. 3.23 Total proteases at pH 1.5 and 3.0 in the stomach of rainbow trout fed for 2 months with 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

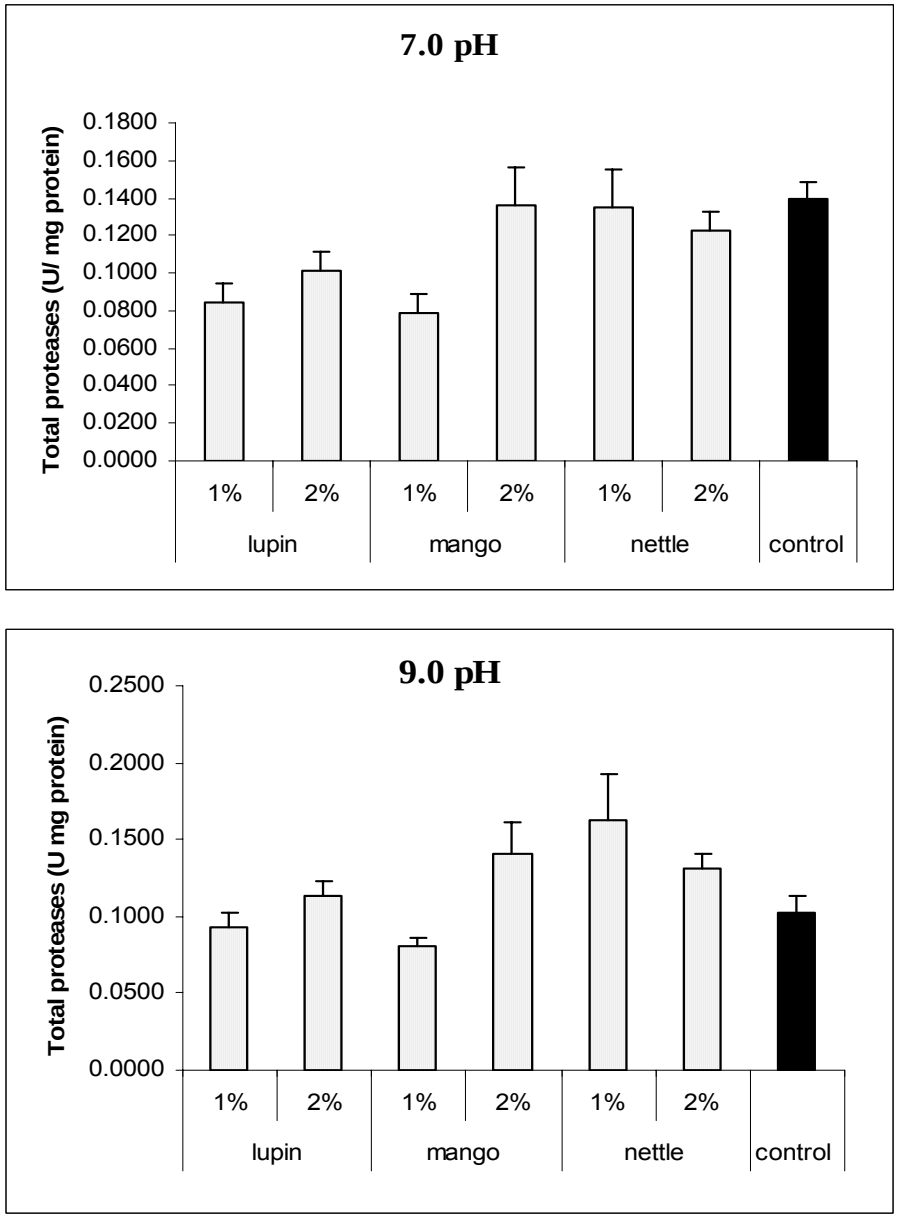


Fig. 3.24 Total proteases at pH 7.0 pH and 9.0 in the intestine of rainbow trout fed for 2 months with 1% and 2% lupin, mango and stinging nettle.

Bars = mean ± S.E.

3.8.3 Amylase

In the stomach, feeding with 2% lupin and 2% stinging nettle for 2-months resulted in 0.122 and 0.267 U/mg protein compared to 0.017 and 0.029 U/mg protein in the fish which received 1% of the plant material (Fig. 3.25). In comparison 1% mango led to higher amylase activity than the 2% dose. Moreover, 2% of all three dietary supplements led to higher amylase activity in the intestine compared to the 1% dose (Fig. 3.25). Amylase activity in the controls was 0.012 and 0.031 U/mg protein in the stomach and intestine, respectively. However, the data were not statistically significant.

3.8.4 Lipase

There was not any significant difference between control and treated groups in the stomach and intestine in terms of lipase activity. Nevertheless, in the stomach, 2% lupin and stinging nettle resulted in 2.42 and 17.78 U/mg protein (Fig. 3.26), which was higher than the 1% dose (1.27 and 6.44 U/mg protein, respectively). Conversely, the higher lipase activity resulted with feeding 1% rather than 2% mango (3.16 U/mg protein for the 1% dose in the stomach). The controls revealed 0.83 U/mg protein. In the intestine, the highest lipase activity was recorded with the 2% dose of all three plant materials (Fig. 3.26).

3.8.5 Pepsin

Generally, pepsin activity was highest in the stomach rather than the intestine (Fig. 3.27). In stomach, the 2% dose of lupin and stinging nettle led to higher pepsin activity than the 1% amount. Conversely with mango, the 1% dose resulted in higher pepsin activity than the 2% amount. Opposite observation was recorded in intestine. Moreover, the controls recorded the lowest value in both stomach and intestine (19.45 and 0.18 U/mg protein, respectively). In stomach the data were significantly different ($p < 0.05$) for the 2% dose of lupin and stinging nettle and with 1% mango (169.82, 170.25 and 127.91 U/mg protein, respectively).

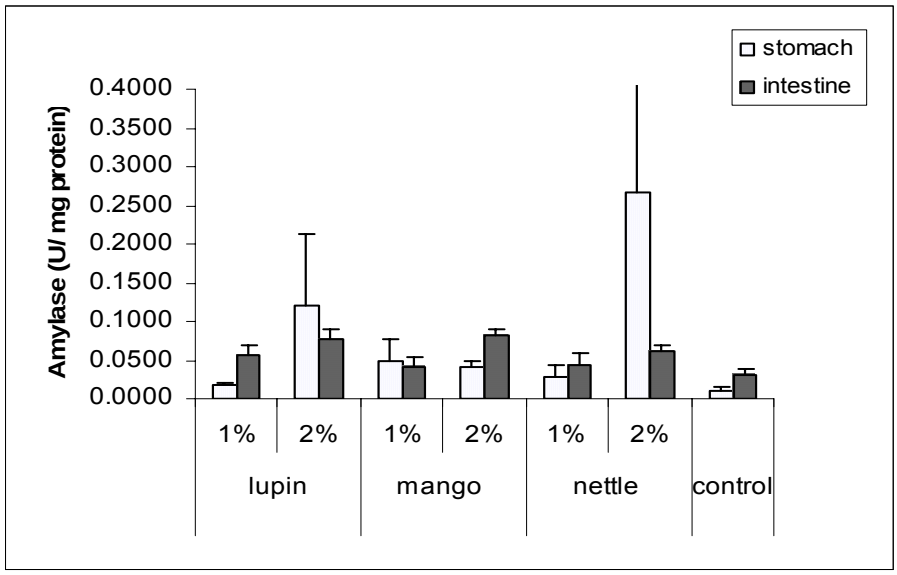


Fig. 3.25 Amylase activity in stomach and intestine of rainbow trout fed for 2 months with 1% and 2% lupin, mango and stinging nettle.

Bars = mean ± S.E.

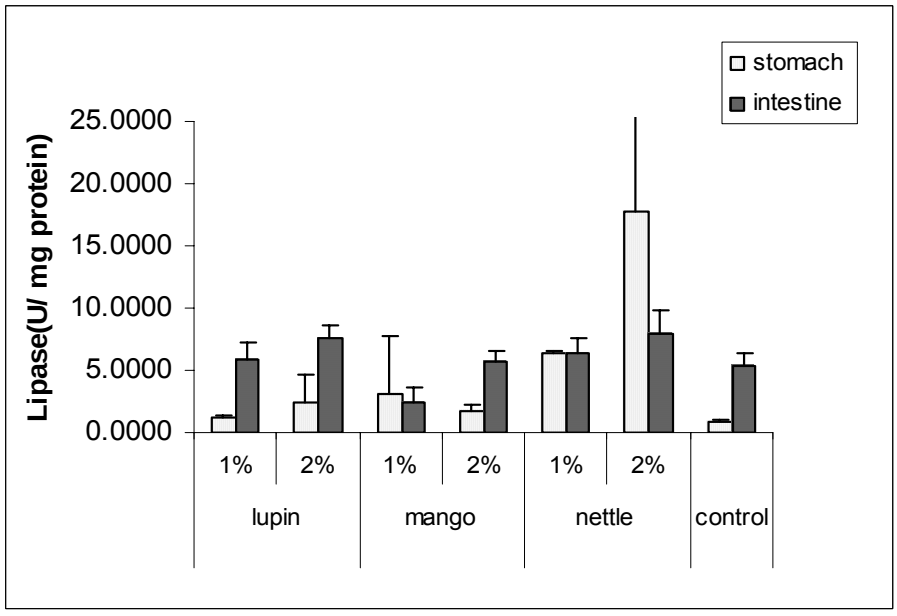


Fig. 3.26 Lipase activity in stomach and intestine of rainbow trout fed for 2 months with 1% and 2% lupin, mango and stinging nettle.

Bars = mean ± S.E.

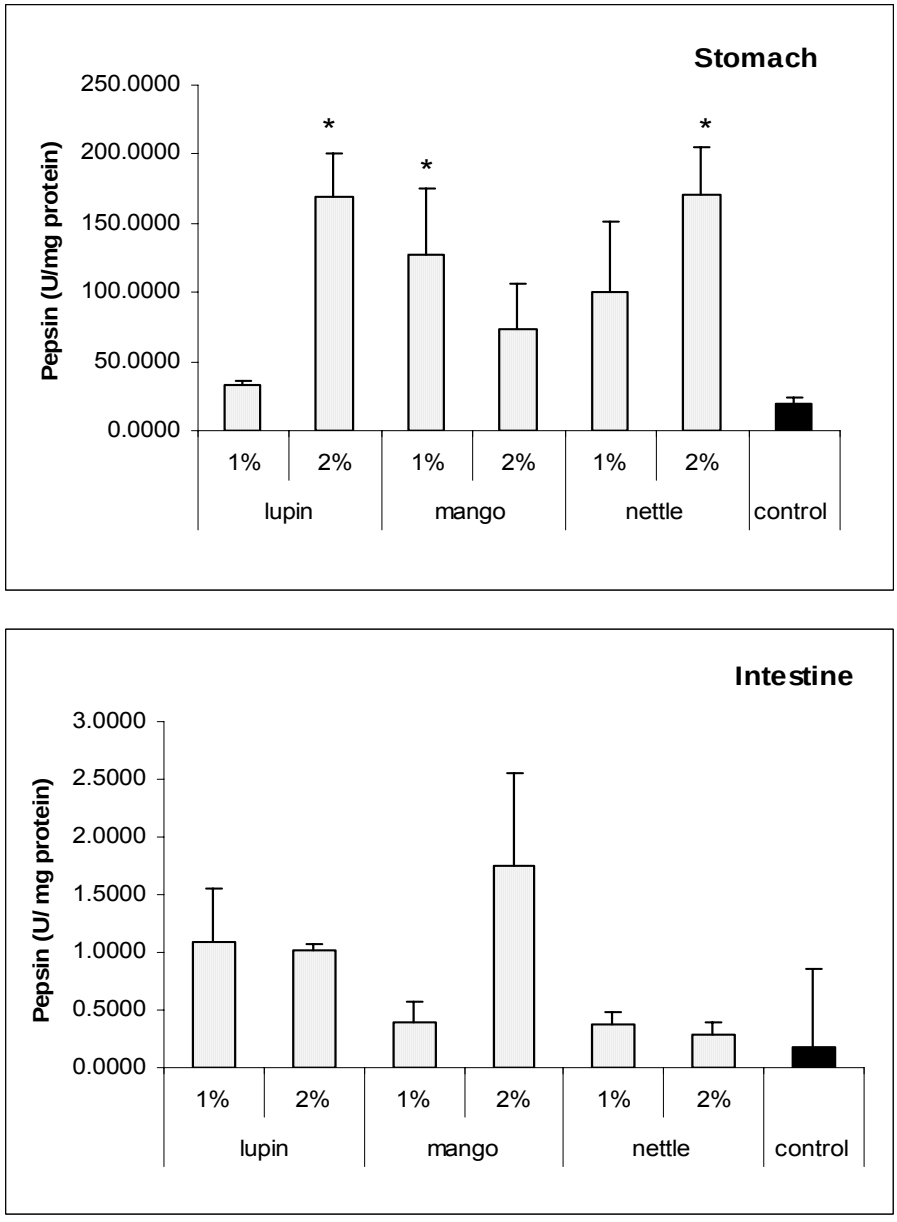


Fig. 3.27 Pepsin in stomach and intestine of rainbow trout fed for 2 months with 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.9 Effect of long term feeding with dietary supplements on growth performance

The final weight, weight gain and specific growth rate (SGR) of the controls showed the lowest significant values ($p < 0.05$). In contrast, feeding with 1% stinging nettle and mango led to the highest level than 2%. Conversely with lupin, the 2% dose resulted in a higher value than the 1% dose (Table 3.5). However, feeding with 1% lupin, mango and stinging nettle for 2 months led to the highest length and condition factor compared to the controls ($p < 0.05$).

For the feed conversion ratio (FCR), the results for 1% lupin, mango and stinging nettle were higher than the corresponding 2% doses. Moreover, the control did not show the lowest value; instead this was recorded with 2% of lupin, which was significantly different to data with 1% lupin (the highest value). The controls showed the lowest value in terms of feed intake, without a significant difference compared to treatments. The 2% doses of stinging nettle and mango were higher than the 1% doses in terms of feed intake. Conversely, 1% lupin led to higher values than the 2% dose. Yet, there was not any significant difference between any of the data. In addition, 2% of mango, stinging nettle and control were the highest in terms of the day intake ratio (DIR).

Table 3.5 Growth performance of rainbow trout fed for 2 months with dietary supplement of 1% and 2% lupin, mango and stinging nettle

	Dose	**final weight**	**weight gain**	**SGR**	**length**	**CF**	**FCR**	**feed intake**	**DIR**
		g	g	%	cm	g/cm	%	g/fish	
	1%	48.81 ± 0.63*	30.81± 0.63*	0.51 ± 0.01*	15.9 ± 0.14*	3.07 ± 0.03	2.01 ± 0.04	23.16 ± 0.01	0.79 ± 0.01
Lupin	2%	49.39 ± 1.02*	31.39 ± 1.02*	0.52 ± 0.02*	15.67 ± 0.04*	3.15 ± 0.06	1.42 ± 0.05	22.17 ± 0.1	0.75 ± 0.02
	1%	49.17 ± 0.93*	31.17 ± 0.93*	0.52 ± 0.02*	15.77± 0.04*	3.12 ± 0.06	1.90 ± 0.06	22.16 ± 0.05	0.75 ± 0.01
Mango	2%	45.52 ± 1.09*	27.53 ± 1.09*	0.46 ± 0.004*	15.37 ± 0.11*	2.96 ± 0.05	1.85 ± 0.02	25.44 ± 0.06	0.93 ± 0.02
	1%	50.34 ± 0.27*	32.34 ± 0.27*	0.54 ± 0.01*	16.11 ± 0.05*	3.12 ± 0.01	1.75 ± 0.05	21.29 ± 0.02	0.71 ± 0.01
Nettle	2%	49.55 ± 0.53*	31.55 ± 0.53*	0.53 ± 0.008*	15.9 ± 0.04*	3.12 ± 0.03	1.56 ± 0.03	24.63 ± 0.03	0.83 ± 0.01
Control		36.9 ± 2.46	20.9 ± 2.46	0.35 ± 0.05	14.63 ± 0.31	2.52 ± 0.12	1.82 ± 0.05	20.91 ± 0.03	0.87 ± 0.06

Data represented as mean ± SE.

(*) = Significant difference from control $p < 0.05$

3.10 Effect of long term feeding with dietary supplements on body composition

The crude protein levels for whole dried fish were highest after feeding with 1% and 2% of lupin, and 1% of mango compared to controls (Table 3.6). In whole dried fish, the lipid was higher after administering 1% than 2% lupin, mango and stinging nettle. Although, the controls revealed the lowest levels, there was not any significant difference between each treatment group and the controls. With moisture and ash, 2% stinging nettle and mango led to higher levels than the corresponding 1% doses. The reverse occurred with lupin when 1% led to higher levels of moisture and ash than the 2% dose. Again, there was not any significant difference between the treatment and control groups.

Table 3.6 Composition of the whole body of rainbow trout after feeding for 2 months with dietary supplement of 1% and 2% lupin, mango and stinging nettle

	Dose	Crude protein	Crude Lipid	Moisture	Ash
	1%	62.45 ± 6.74	34.42 ± 2.58	72.34 ± 0.56	7.94 ± 0.89
Lupin	2%	58.67 ± 3.27	32.25 ± 0.37	70.02 ± 1.18	7.25 ± 0.47
	1%	58.98 ± 2.03	35.28 ± 0.43	68.12 ± 4.43	7.34 ± 0.13
Mango	2%	56.5 ± 3.5	28.75 ± 0.92	72.77 ± 0.68	7.47 ± 0.53
	1%	56.8 ± 1.58	32.55 ± 1.7	72.33 ± 0.89	7.87 ± 0.47
Nettle	2%	56.28 ± 1.68	32.21 ± 1.39	72.73 ± 0.55	8.35 ± 0.55
Control		57.54 ± 1.04	27.77 ± 2.11	72.97 ± 0.34	8.62 ± 0.4

Data represented as mean ± SE.

3.11 Effect of long term feeding dietary supplements on electrolytes in the plasma

Data for plasma electrolytes of rainbow trout fed with 1% and 2% lupin, mango and stinging nettle for 2 months are included in Table 3.7. Plasma sodium (Na) was higher in the group fed with 2% mango and 2% stinging nettle than with the 1% dose. The opposite was true in lupin. Here, the lowest value was recorded in the 2% dose of lupin and the 1% dose of stinging nettle, which showed significant differences with other groups and the controls ($p < 0.05$). The highest value of plasma potassium (K) was in the control, but K was slightly higher in lupin compared with mango and stinging nettle fed fish. Generally, 2% doses led to higher levels than 1% doses, with no significant difference between any of groups and the controls. The lowest value in plasma calcium (Ca) was recorded in the controls. However, 2% lupin and 2% stinging nettle led to higher levels than the 1% dose, and *vice versa* in the case of mango. There was not any significant difference observed between any treatment group and the controls. Plasma magnesium (Mg) levels in fish fed with 2% lupin, mango and stinging nettle were higher than the 1% doses, but less than the controls. The only significant difference was between 2% and 1% lupin, the latter of which revealed the lowest value ($p < 0.05$). The 2% doses of all three plants led to higher plasma zinc (Zn) values than the 1% doses. Similarly, plasma iron levels in fish which received 2% of mango and stinging nettle were higher than those fish which received the 1% doses. The highest significant value was recorded in fish which received 1% lupin ($p < 0.05$), whereas the lowest value was in the controls.

Table 3.7 Electrolytes in rainbow trout after feeding for 2 months with 1% and 2% lupin, mango and stinging nettle

	Dose	Na	K	Ca	Mg	Zn	Iron
Lupin	1%	111.61 ± 0.05	0.83 ± 0.42	3.86 ± 0.61	0.96 ± 0.08	1.46 ± 0.02	1.58 ± 0.01*
	2%	72.15 ± 2.47*	0.87 ± 0.29	3.98 ± 0.25	1.75 ± 0.28	1.61 ± 0.02	1.16 ± 0.02
Mango	1%	102.10 ±1.98	0.63 ± 0.05	4.52 ± 0.56	1.21 ± 0.06	1.53 ± 0.02	1.14 ± 0.04
	2%	103.26 ± 0.61	0.66 ± 0.19	3.83 ± 0.25	1.29 ± 0.18	1.62 ± 0.06	1.47 ± 0.01*
Nettle	1%	74.90 ± 0.75*	0.49 ± 0.02	3.58 ± 0.17	1.21 ± 0.06	1.51 ± 0.02	1.16 ± 0.02
	2%	121.79 ± 10.7	0.66 ± 0.02	4.29 ± 0.49	1.54 ± 0.20	1.48 ± 0.06	1.21 ± 0.01
Control		101.26 ± 1.88	1.46 ± 0.28	3.31 ± 0.24	1.37 ± 0.12	1.29 ± 0.03	0.94 ± 0.01

Data represented as mean ± SE.

(*) = Significant difference from control $p < 0.05$

3.12 Effect of long term feeding of dietary supplements on physiological parameters

3.12.1 Liver function

Serum GOT for fish fed for 2 months with 1% mango and stinging nettle (413.75 and 375.22 U/L, respectively) were higher than the 2% doses (387.56 and 374.42 U/L, respectively) (Fig. 3.28). Conversely, use of 1% and 2% lupin led to similar data. Generally, the controls had the lowest significant value (355.20 U/L) ($p < 0.05$). Fish fed with 1% mango (319.97 U/L) (Fig. 3.28) had higher GPT values, more so than animals which received the 2% dose (300.99 U/L). Conversely, 2% stinging nettle (300.33 U/L) led to higher readings than the 1% dose (298.34 U/L). Lupin gave almost the same results at both doses. The controls had the lowest significant value (283.01 U/L) ($p < 0.05$).

3.12.2 Kidney function

Fish, which received 2% mango and stinging nettle revealed higher levels of urea (8.50 and 7.17 mg/dl, respectively) than the 1% dose (7.39 and 5.33 mg/dl, respectively) (Fig. 3.29). In contrast, urea in fish which received 1% lupin (8.11 mg/dl) was higher than those animals fed with the 2% dose (7.39 mg/dl). Although controls had the highest amount of urea (9.94 mg/dl), there was not any significant difference with any of the treatment groups. Also, the controls had the highest amount of creatinine (1.15 mg/dl), ($p < 0.05$) (Fig. 3.29). Of the treatment groups, rainbow trout fed with 1% doses of lupin, mango and stinging nettle had the highest levels of creatinine (0.64 and 0.76, 0.45 mg/dl, respectively), more than the 2% doses.

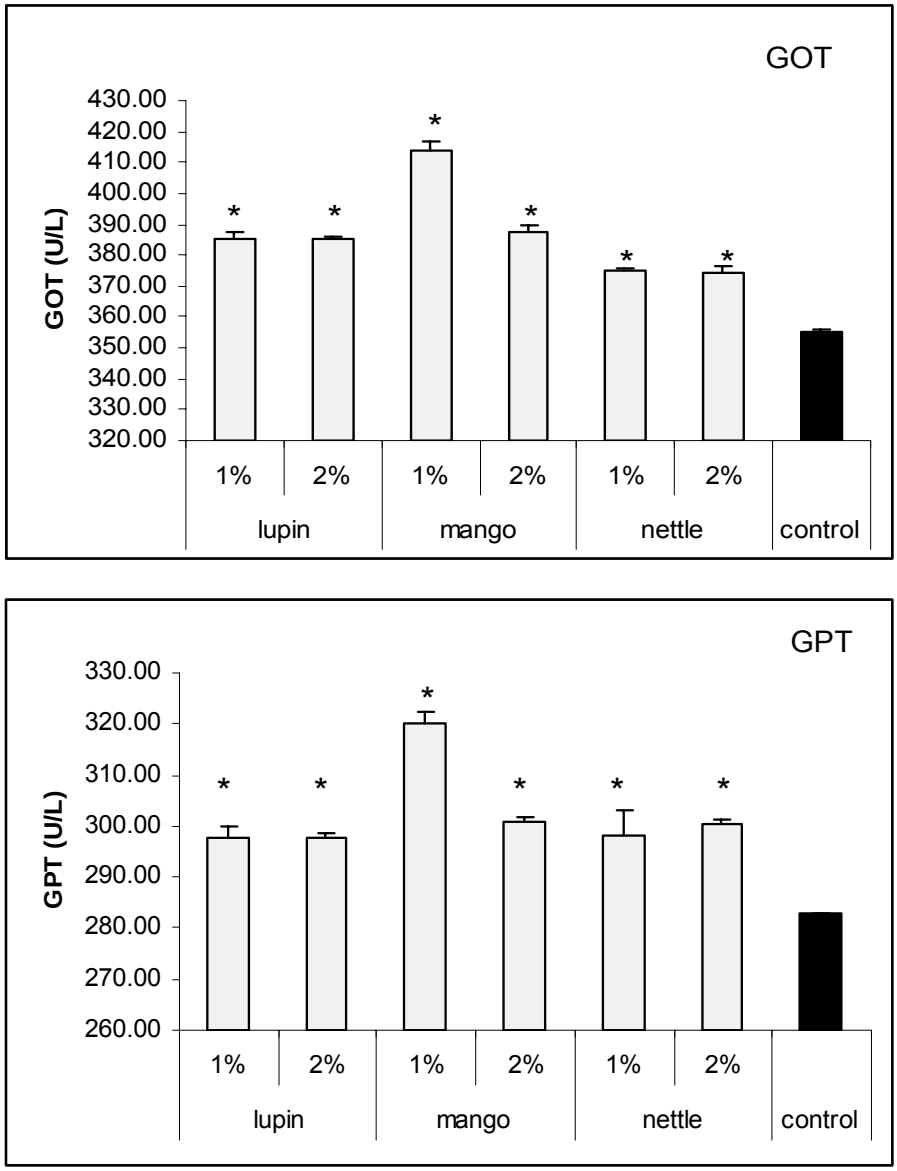


Fig. 3.28 Serum GOT and GPT of rainbow trout fed for 2 months with 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

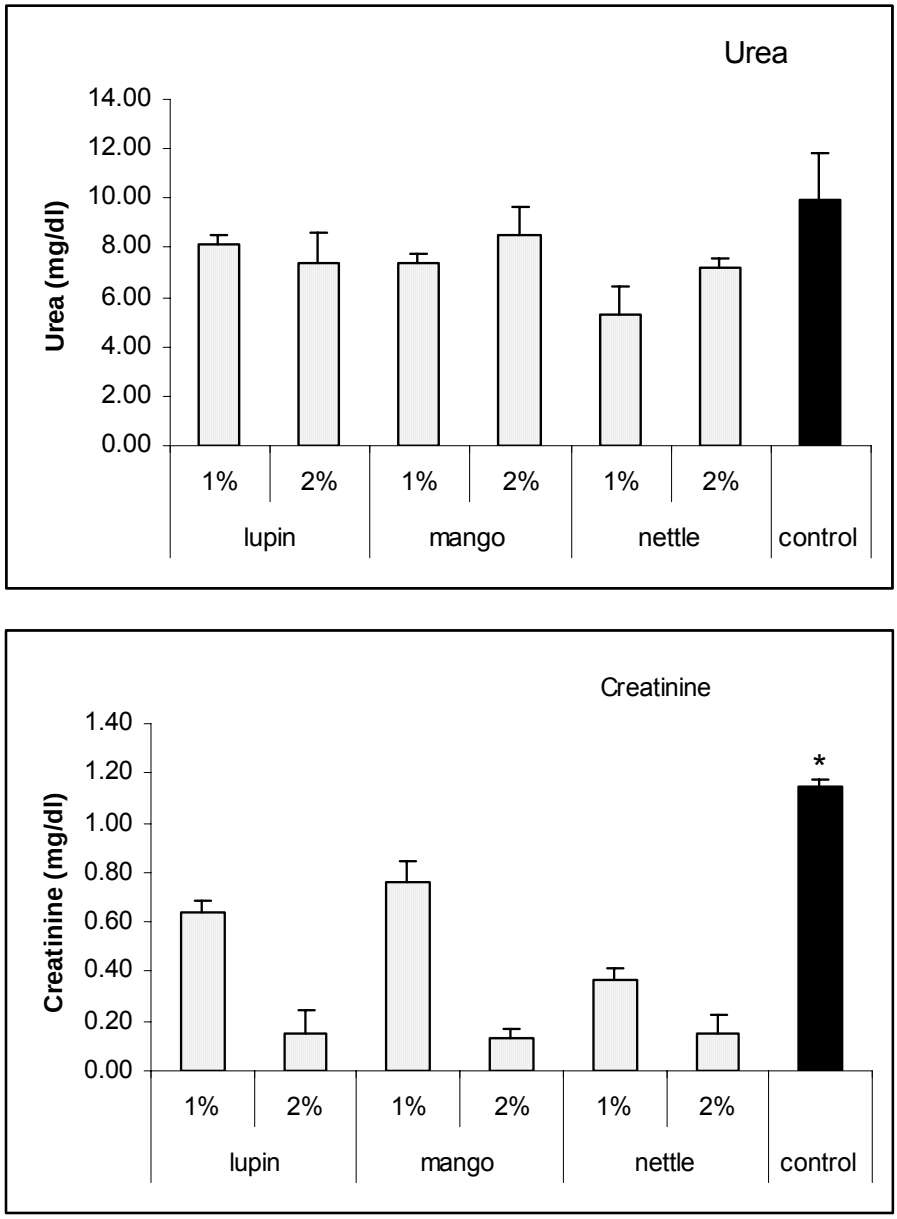


Fig. 3.29 Serum urea and creatinine of rainbow trout fed for 2 months with 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.12.3 Glucose

Serum glucose was higher in fish fed with 1% doses of lupin, mango and stinging nettle (263.5, 125.56 and 162 mg/dl, respectively) compared to the 2% doses (92.52, 106.03 and 116.52 mg/dl, respectively) (Fig. 3.30). The controls had the lowest significant value (62.94 mg/dl) ($p < 0.05$). Interestingly, 1% lupin (263.5 mg/dl) recorded the highest value of all the treatment groups.

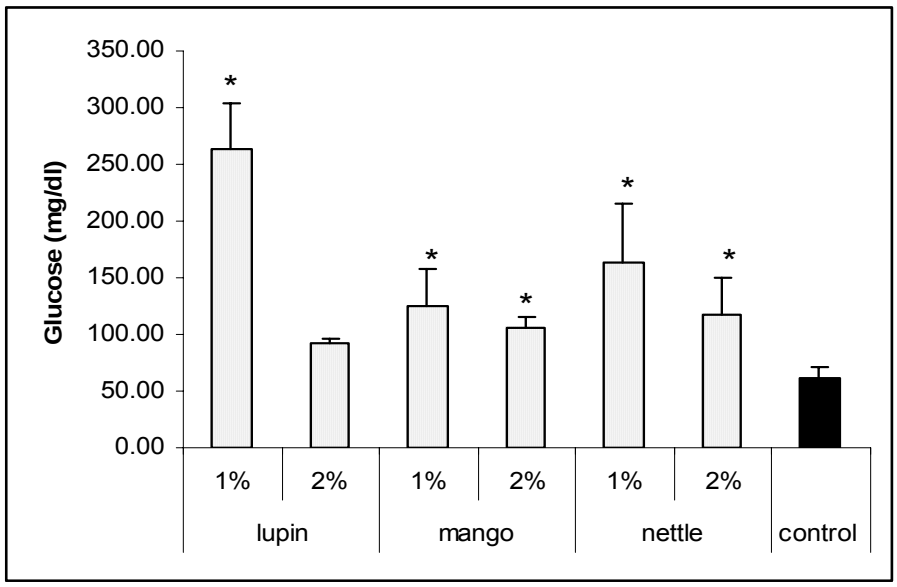


Fig. 3.30 Serum glucose of rainbow trout fed for 2 months with 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.12.4 Total protein

Feeding for 2 months led to high protein values in all the treatment groups, with significant differences compared to control (3.34 g/dl) ($p < 0.05$). Generally, 1% lupin and 1% stinging nettle led to higher values (7.16 and 5.9 g/dl) than the 2% doses (4.76 and 4.36 g/dl), and *vice versa* in the case of mango. Interestingly, 1% of lupin (7.16 g/dl) recorded the highest significant compared with all treated groups (Fig. 3.31).

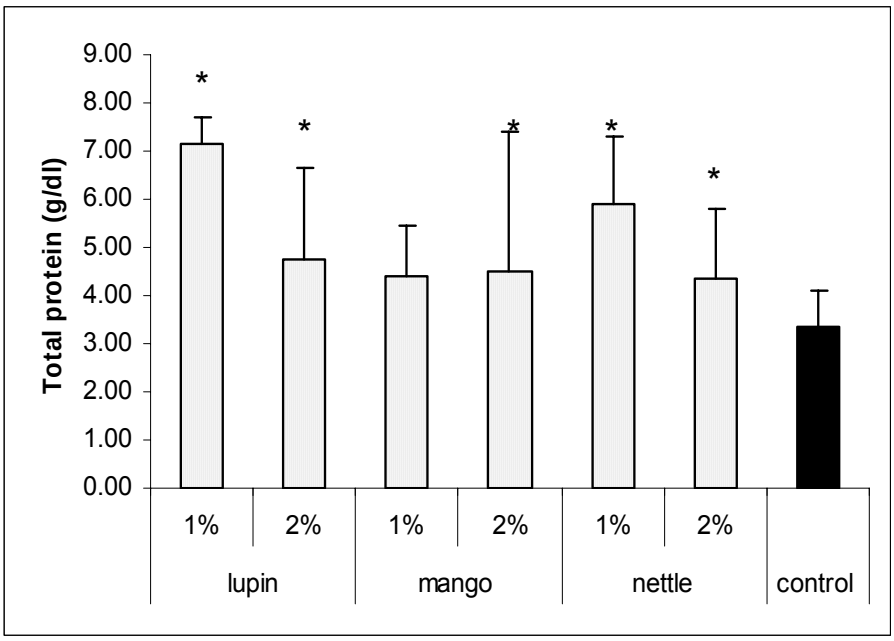


Fig. 3.31 Total protein of rainbow trout fed for 2 months with 1% and 2% lupin, mango and stinging nettle.

(*) = Significant difference from control $p < 0.05$

Bars = mean ± S.E.

3.13 Effect of long term feeding with dietary supplements on cellular immune responses

3.13.1 Red blood cell (RBC) and white blood cell (WBC) count

The RBC count was higher in fish fed for 2 months with 1% lupin, mango and stinging nettle, more so than the 2% doses (Table 3.8). Fish fed with 1% lupin and 1% mango revealed higher numbers of WBC than the 2% doses. However, all the treatment groups was higher than the control in terms of RBC and WBC counts, although there was not any significant difference between the treatment groups and the controls ($P > 0.05$).

.

3.13.2 Haematocrit (Hct) and haemoglobin (Hb)

The Hb value in fish fed with 1% lupin and stinging nettle was higher than the 2% doses, and higher than the controls. With mango, the Hb value after both doses was similar (Table 3.8). Interestingly, the Hct values after feeding with 1 % lupin and 1% mango were higher than the 2%, doses, and with highly significant differences in the data ($p < 0.05$). In contrast, feeding with 2% stinging nettle led to higher levels than achieved with the 1% dose.

Table 3.8 RBC, WBC, Hct and Hb of rainbow trout fed for 2 months with 1% and 2% lupin, mango and stinging nettle.

	Dose	RBC x 10^6/µl	WBC x 10^4/µl	Hb (g/dl)	Hct (%)
Lupin	1%	1.02 ± 0.13	9.14 ± 3.14	13.51 ± 0.41	41.62 ± 0.53
	2%	0.91 ± 0.07	7.15 ± 1.37	10.81 ± 0.70	34.45 ± 1.78*
Mango	1%	1.26 ± 0.18	9.4 ± 3.39	16.05 ±1.48	47.88 ± 1.21
	2%	1.15 ± 0.05	8.51 ± 0.94	16.09 ± 4.08	30.93 ± 0.78*
Nettle	1%	1.03 ± 0.1	6.75 ± 0.49	12.19 ± 2.46	40.35 ± 2.02
	2%	0.96 ± 0.12	8.32 ± 1.92	10.81 ± 0.67	42.96 ± 1.31
Control		0.86 ± 0.04	4.83 ± 0.37	9.40 ± 2.13	41.06 ± 0.53

Data represented as mean ± SE.

(*) = Significant difference from control $p < 0.05$

3.13.3 Differential leucocyte and thrombocyte counts

Data for differential leucocyte and thrombocyte counts for fish fed for 2 months with 1% and 2% lupin, mango and stinging nettle are included in Table 3.9. The highest numbers of lymphocytes were recorded in the controls and there was not any significant difference with treatment groups. The administration of 2% mango and 2% stinging nettle revealed higher lymphocyte counts than the 1% doses. The opposite was true of lupin. For monocytes, all the treatment groups gave higher numbers than the controls. Also, 1% mango and 1% stinging nettle led to higher counts than the 2% doses. With lupin, the two doses led to similar counts.. However, there was not any significant difference between the controls and treatments groups. With exception of 1% lupin, all the treatment groups led to neutrophil counts higher than the controls, especially with 2% mango which revealed highly significant differences with the control. Both doses of lupin and mango led to similar thrombocyte counts. However, use of 2% stinging nettle led to higher counts than the 1% dose, but both of them were less than the controls. There was not any significant difference between control and treatment groups.

Table 3.9 Leucocyte and thrombocyte counts in rainbow trout fed for 2 months with 1% and 2% of lupin, mango and stinging nettle.

	Dose	Lymphocytes	Monocytes	Neutrophils	Thrombocytes
Lupin	1%	93 ± 1.45	3 ± 0.33	1 ± 1	3 ±1.2
	2%	92 ± 0.88	3 ± 0.33	2 ± 0.58	3 ± 1.15
Mango	1%	90 ± 2	3 ± 0.67	5 ± 1.45*	2 ± 0.33
	2%	94 ± 1.45	2 ± 1	2 ± 0.33	2 ± 0.58
Nettle	1%	92 ± 2.08	5 ± 1.86	2 ± 1	1 ± 0.33
	2%	93 ± 1.45	3 ± 1.20	2 ± 1	2 ± 0.67
Control		95 ± 0.88	1 ± 0.33	1 ± 1	3 ± 0.58

Data represented as mean ± SE.

(*) = Significant difference from control $p < 0.05$

3.14 Cytokine gene expression in head kidney induced by dietary supplements

3.14.1 RNA and PCR products

rRNA was run on gels in order to evaluate the quality of RNA before preparing cDNA (Fig 3.32). PCR products of each gene (Table 2.2) were amplified at 58^{o}C and 61^{o}C (of annealing temperature) for reference gene (ß-actin) and the target genes (IL-8, IL-1ß and TGF-ß1) were 186, 162, 181, and 275 bp respectively (Fig. 3.33).

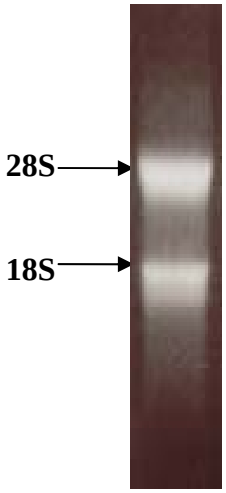


Fig 3.32 rRNA product on 1% agarose-MOPS- formaldehyde gel showing18S and 28S bands

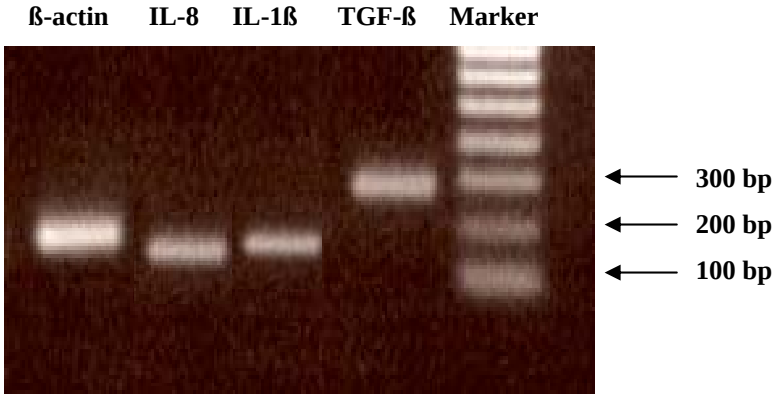


Fig 3.33 PCR product of reference gene (ß-actin) and the target genes (IL-8, IL-1ß and TGF-ß1), respectively. Marker of 1 kps (Hyper ladder 1; Bioline) was used to evaluate the gene size. 300, 200 and 100 correspond to bp values as determined from the marker.

3.14.2 PCR efficiency

The PCR efficiency was determined for Real-time PCR performance for each primer using a 10 fold serial dilution of cDNA. The figure (Fig 3.34) shows plots of threshold cycle (C_T) versus the logarithmic cDNA input, using the equation (Rasmussen, 2001):

$E = -1 + 10^{(-1/\text{slop})}$

The PCR efficiencies were 1.94, 1.95, 2.02 and 1.99 for ß-actin, IL-8, TGF-ß1 and IL-1ß, respectively (Fig. 3.34).

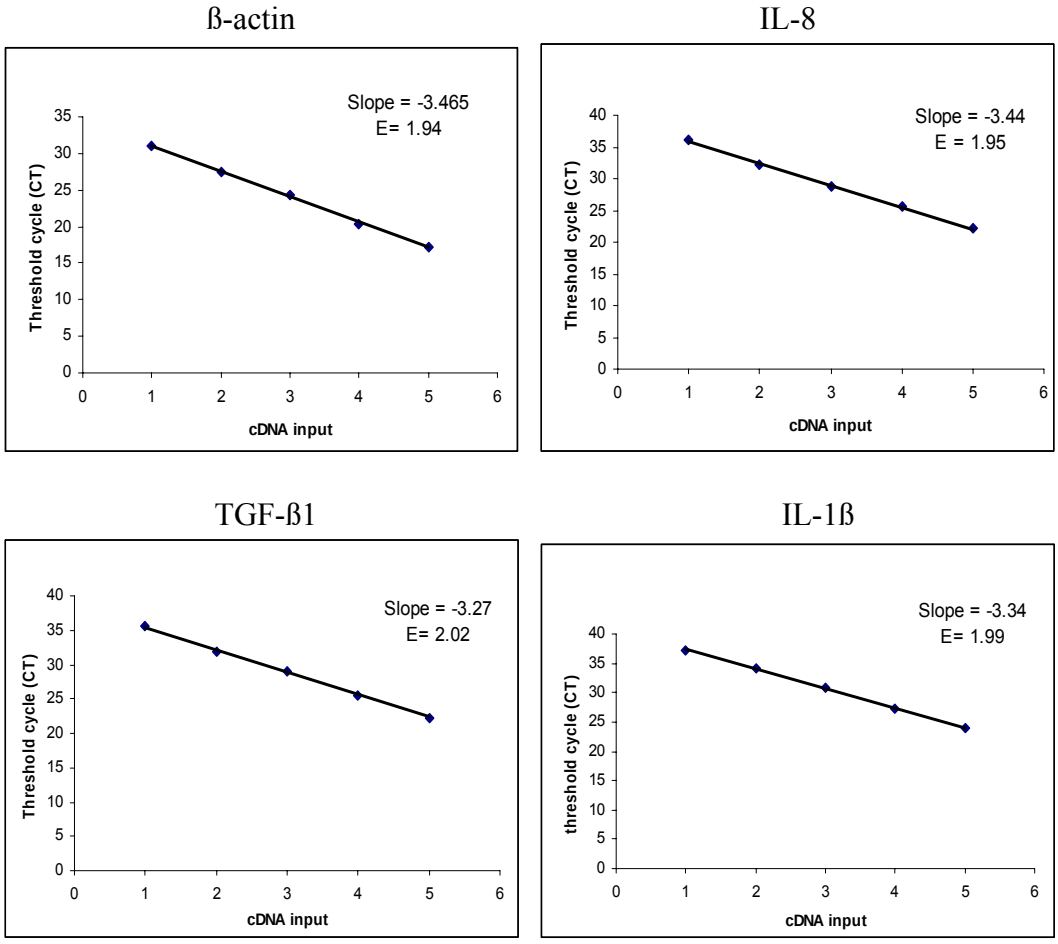


Fig. 3.34 Real-time PCR efficiencies of reference gene (ß-actin) and the target genes (IL-8, TGF-ß1 and IL-1ß). The slope was calculated from plot threshold cycles (C_T) versus cDNA input. The efficiencies (E) were calculated from the slope according to the above mentioned equation.

3.14.3 Melt curve

The melt curve was calculated by iCycler™ software. At the beginning, the temperature was raised by a fraction of a degree, and the change in fluorescence was measured (Fig. 3.35). The melting point happened when the two strands of DNA separate and the fluorescence rapidly decreases. The software plots the rate of change of the relative fluorescence units (RFU) with time (T) (-d(RFU)/dT) versus the temperature, and this peaked at the melting temperature (T_m). The T_m was 91, 82, 89 and 89°C for ß-actin, IL-8, TGF-ß1 and IL-1ß, respectively.

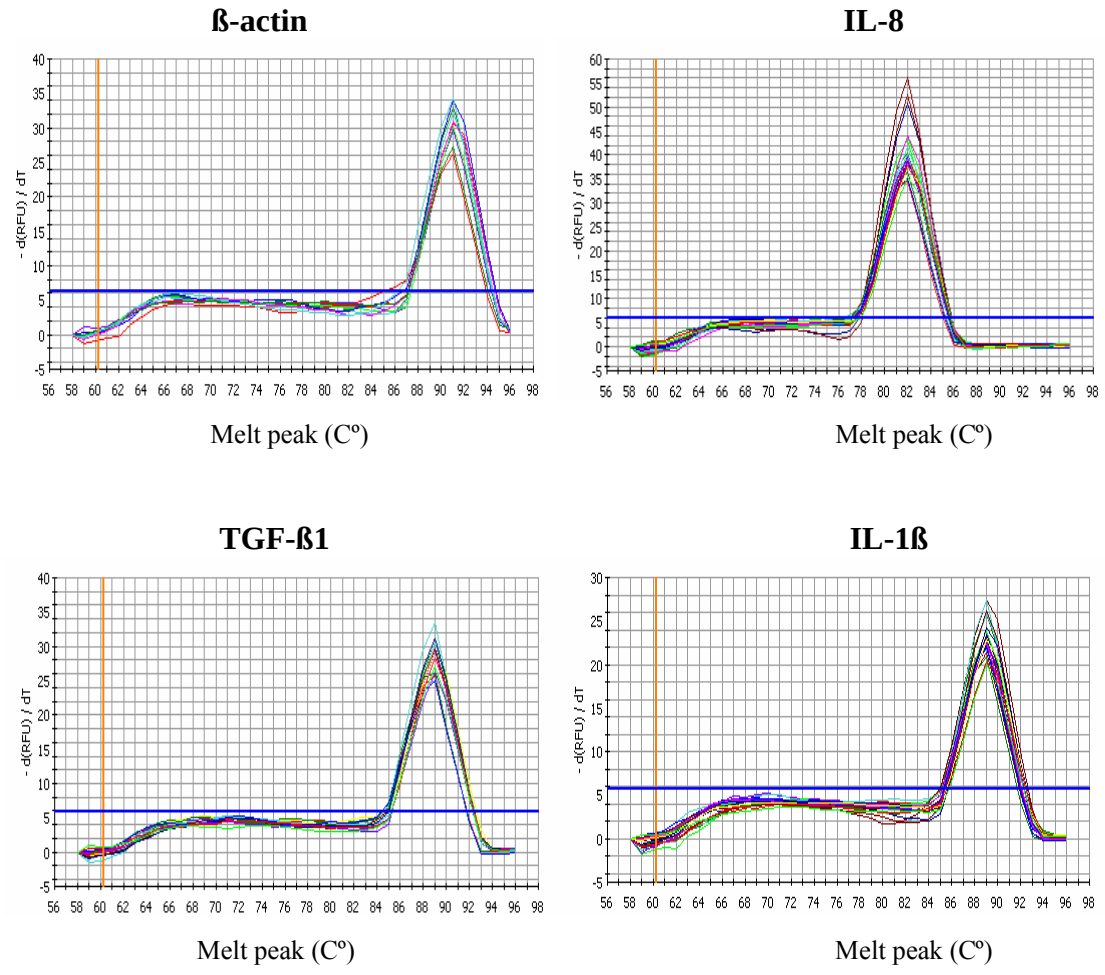
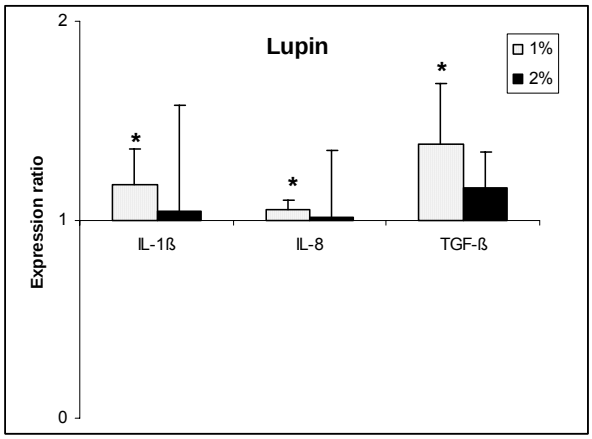
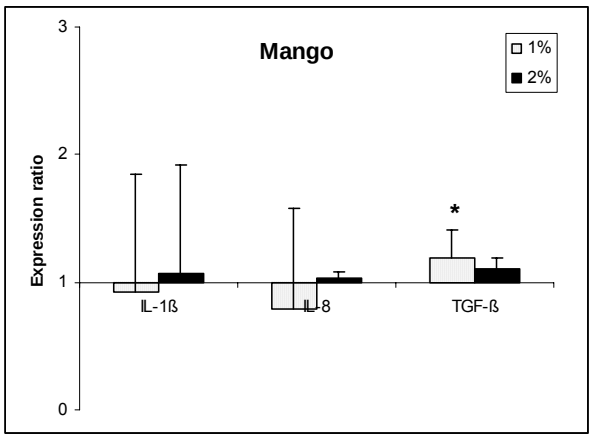


Fig. 3.35 Melt curve for reference gene (ß-actin) and the target genes (IL-8, TGF-ß1 and IL-1ß).

3.14.4 Cytokine expression in head kidney

The expression ratio of target genes (IL-8, IL-1ß, and TGF-ß1) of head kidney of fish fed with 1% and 2% lupin, mango and stinging nettle have been included in Fig. 3.36. With lupin, the results showed up-regulation in the target genes as compared to the control. The expression following the 1% dose of lupin was significantly different ($p < 0.05$). Although the expression of target genes in the group fed with 2% mango was higher than the control, the data was not statistically significant. Moreover, the expression ratio in the group fed with 1% mango was lower than the control (down-regulation) except in the case of TGF-ß1 which was statistically significant different ($p < 0.05$). Interestingly, the expression ratio of target genes for both doses of stinging nettle was upregulated and significant when compared with the controls.





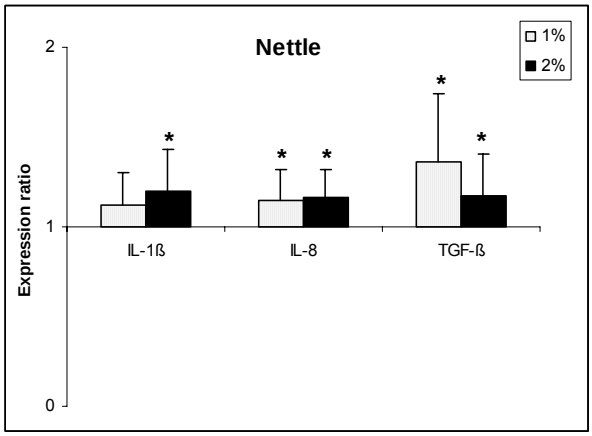


Fig. 3.36 Expression ratio for IL-1ß, IL-8 and TGF-ß1 in head kidney of rainbow trout after feeding with 1% and 2% lupin, mango and stinging nettle for two months. Data are represented as expression ratio ± SE and, and were compared to controls by using the Pair Wise Fixed Reallocation Randomisation Test ©. (*) = $P < 0.05$.

Discussion

Aquaculture production has increased steadily during the years since the end of World War 2, and in many countries particularly in the developing world, the activity is viewed as an important source of high quality protein for local consumption. Not only is there an increase in overall production but there is evidence for a diversification of species farmed and for increasing reasons including encompassing the high end consumer market and biotechnology. Intensive fish culture is subjected to stressors, such as overcrowding, handling, transport, and poor water quality (Li *et al.,* 2004). This interaction between stressor, host and disease-causing agents are the main reasons for the occurrence of disease outbreaks (Austin & Austin, 2007). Bacterial diseases are arguably among the most significant to aquaculture (Sano, 1998).

Infections caused by members of the genus *Aeromonas*, which have a relatively high antibiotic resistance, are among the most common bacterial diseases of fish (Saavedra *et al*., 2004). *A. hydrophila* is one of the most common aerobic heterotrophic bacteria in freshwater and occasionally in the marine environment, including fish (Lilley *et al.,* 1997; Cipriano *et al*., 2001; Pianetti *et al*., 2005). The organism has been associated with a range of diseases in fish and humans; the latter via contaminated food or drink (Handfield *et al.,* 1996) or sometimes by direct contact with contaminated water. In fish, *A. hydrophila* is associated with a range of conditions, including dermal ulceration, tail or fin rot, haemorrhagic septicaemia, scale protrusion and distended abdominal disease (Cipriano *et al*., 2001; Shao *et al.,* 2004). In humans, the organism causes acute diarrhoea (Juan *et al*., 2000; Subashkumar *et al.,* 2007), septic arthritis (Cipriano *et al*., 2001) corneal ulcers, skin and wound infections, meningitis, fulminating septicaemias (Von Gravenitz & Mensch 1968; Davis *et al.,* 1978), and pyaemia associated with fever, rigours and abdominal bloating (Tulsidas *et al*., 2008). However, there is a problem insofar as traditionally motile aeromonads associated with fish diseases have inevitably been regarded as *A. hydrophila,* often after comparison of only a comparatively few phenotypic characters (Austin & Austin, 2007). More recently, some older cultures have been re-assessed taxonomically with the realization that misidentification had occurred (Abbass *et al.,* 2010). The outcome was the appreciation that a wider range of *Aeromonas* spp. were associated with fish diseases. Notwithstanding, the isolate

examined in this study was regarded as a *bona fide* representative of *A. hydrophila,* the identity of which had been confirmed by colleagues using 16S rRNA sequencing. The reason for studying disease control with this organism reflected the dominance of motile aeromonads as causal agents of septicaemic conditions in farmed freshwater fish and humans in developing countries, including Egypt (Ibrahem *et al.*, 2008**;** Ghanem *et al.,* 1993).

The history of aquaculture contains much information about the methods used to control disease. After World War 2 with the increasing use of antibiotics in human medicine, aquaculture followed suit with an ever greater range of chemotherapeutants used to control bacterial diseases (Austin & Austin, 2007). Therefore, the underlying strategy was the control rather than prevention of actual diseases. Undoubtedly, the approach worked initially. However, the extensive use of chemotherapeutants led to the inevitable emergence of drug-resistant bacterial strains (Wooley *et al.,* 2004), and thus a diminution in effectiveness. Moreover, there was the added concern about the accumulation of these bioactive substances in the environment, their spread up the food chain, and the concomitant spread of antibiotic-resistant strains to other organisms of human and veterinary significance (Aruna & Chandran, 1996). Issues with resistance undoubtedly led to a resurgence of interest in preventative, i.e. prophylactic, approaches to disease control, initially by the use of vaccination (Austin & Austin, 2007). Although use of fish vaccines have become an established and effective method of controlling fish diseases, they are comparatively costly involving large developmental and licensing sums, are generally effective only against the specific pathogen and not against variants that might occur from time to time, and are available for only a comparatively few major diseases, including enteric redmouth, furunculosis and vibriosis (Harikrishnan & Balasundaram, 2005). Many serious pathogens have not been targeted for commercial vaccine production. In the case of *A. hydrophila,* the comparative lack of interest in vaccine development may reflect the uncertainly regarding identification of isolates and the seemingly complex antigenic structure (Ardó *et al.,* 2008). Nevertheless, additional means of disease control have been researched, including use of non specific immunostimulants, probiotics and dietary supplements, e.g. medicinal plants/herbs. Immunostimulants, including ß-1,3-glucans, have become widely accepted as a means of disease prevention (Austin & Austin, 2007), and many commercial fish diets contain the compound. Probiotics are gaining interest particularly in developing countries, and

the mode of action has been considered to include stimulation of innate immunity as well as the more widely accepted method of competitive exclusion (Irianto & Austin, 2002). There is an extensive literature, especially from China **(**Yin *et al.,* 2009**;** Galina *et al*., 2009), describing the benefit of a wide range of plants and plant products for disease control. This literature formed the basis of the present study whereby plants were examined for their ability to control infection caused by *A. hydrophila.* The choice of plants was made on the basis of published literature and the availability of material within the Edinburgh area, with the focus on health food shops, supermarkets and Chinese herbalists. The primary reason for this decision was that if anything was proposed for use in aquaculture then it would need to be readily available and reasonably inexpensive. Another consideration was that the plant material would need to be incorporated in an easy a way as possible for it to have any practical value to aquaculture.

4.1 The importance of plants

The use of entire plants or plant products as therapeutic agents has been well known for many years. However with developments in chemical synthesis, much has been done to isolate, characterize and synthesis the bioactive components (Awaad & Al-Jaber, 2010). Natural product chemistry has developed into an important field of research worldwide in the attempt to discover ever more effective and cost effective drugs with lower side effects on the recipients. Although many natural products have milder effects on the body, they have also more positive and longer lasting effects than the synthetic drugs which have faster recovery rates. This may be due to a synergistic effect of several constituents present or reflect a modifying effect by some constituents upon the physical properties of others (Awaad, 2009). There are many plants that possess several biological activities and are currently used in traditional folk medicine (Awaad *et al.,* 2007). Many active plant compounds exert potential immunostimulating activity. For example, there are alkaloids, terpenoids, quinones and phenolic compounds, whereas polysaccharides, peptides, glycoproteins and nucleotides are in the second class (Wagner & Proksch, 1985). Also, many plants produce antioxidant compounds which work as protective agents that inactivate reactive oxygen species and thus delay or prevent oxidative damage, therefore playing major roles in the prevention of diseases (Hudec *et al*., 2007).

Certainly, numerous studies have been carried out on using medicinal plants/herbs in fish to treat bacterial diseases, and also to serve as a cheaper source of nutrients for the animals (Sakai, 1999; Siddhuraju *et al*., 2000; Hossain *et al.*, 2001; Richter *et al*., 2003; Harikrishnan *et al.,* 2003; Rath, 2000). For example, herbs have been used with some success to treat ulcerative dermatitis in common carp; a disease which is caused by *A. hydrophila* (Harikrishnan *et al.,* 2003).

The outcome of the present study is that three plants, i.e. lupin, mango and stinging nettle, were found to control *A. hydrophila* infection in rainbow trout. Lupin is a member of the genus *Lupinus* in the legume family (Fabaceae), which contains ~ 200 species. Lupin seeds are known to have a hypoglycaemic action in diabetic animals (Abdel-Aal *et al*., 1993; Eskander & Won Jon, 1995). Moreover, extracts from lupin seeds have been recorded to exert antidiabetic effect in alloxan diabetic mammals (Abdel-Aal *et al.*, 1993). Antibacterial activity has been reported (Lampart-Szczapa *et al*., 2003). Additionally, lupin protein causes reduction in plasma triglyceride concentrations of rats (Spielmann *et al.,* 2007) and has a hypolipodaemic and anti-atherosclerotic effect in rabbits (Marchesi *et al.,* 2008). Lupin meals are included as ingredients in alternative primary protein sources in aquacultural diets to reduce the amount of fish meal used for fish **(**Burel *et al.,* 1998; Gomes *et al*., 1995; Farhangi & Carter, 2001; Glencross *et al*., 2002; Glencross *et al*., 2007) and shrimp, *Penaeus monodon* (Sudaryono *et al*., 1999)**.** However this is the first report of lupin as a immunostimulant in fish.

Mango (*Mangifera indica*), which belongs to the family Anacardiaceae, is cultivated mainly in tropical and subtropical regions. Different parts of the mango have showed great medicinal potential conferring antimicrobial (Kabuki *et al*., 2000), antiviral, antifungal (Cojocaru *et al.,* 1986), anti-inflammatory (Garrido *et al*., 2004a), anti-diarrhoeal (Sairam *et al*., 2003), antitumor (Garrido *et al*., 2004b) and antioxidant activity (Scartezzini & Speroni, 2002; Anila & Vijayalakshmi, 2003; Maisuthisakul, 2009), as well as immunomodulatory function (Makare *et al*., 2001). In a study aimed at elucidating the active component of mango seed kernel, the presence of polyphenol was recognized (Kabuki *et al*., 2000). This has antimicrobial activity against Gram-positive more so than Gram-negative bacteria (Kabuki *et al*., 2000). Indeed, Sahu *et al*. (2007a) stated that using mango kernel in fish diet stimulated the immunity of rohu, and

increased resistant against *A. hydrophila* infection. Additionally, mango kernel has been used as a feed source for growth and feed utilization of fish (Omoregie *et al*., 1991) and other animals (Okai & Aboagye, 1990; Joseph & Abolaji, 1997).

Stinging nettle is a member of the Urticaceae family, which includes ~ 500 species. The stinging nettle, *Urtica dioica*, is mainly found in Europe, Asia, and North America. The large numbers of active components in stinging nettle encourage its use in medicine for various diseases, including benign prostatic hyperplasia, osteoarthritis**,** hay fever, low blood sugar and blood pressure problems (Koch, 2001; Chrubasik *et al*., 2007). Stinging nettle leaves are rich in flavenoids, chlorophylls and carotenoids and their degradation products, vitamins (Wagner *et al*., 1989; Chaurasia & Wichtl, 1987; Akbay *et al*., 2003), proteins (Dalev *et al.,* 1996), mineral materials, organic acids, and oil (Chaurasia & Wichtl, 1986a, b). Gülçin *et al.* (2004) reported antimicrobial activity in aqueous extracts from stinging nettle with effectiveness against nine micro-organisms (*Pseudomonas aeruginosa, Escherichia coli, Proteus mirabilis, Citrobacter koseri, Enterobacter aerogenes, Staphylococcus aureus, Streptococcus pneumoniae, Micrococcus luteus, Staphylococcus epidermidis* and *Candida albicans*). Also, there was anti-ulcer activity against ethanol-induced ulcerogenesis.

4.2 Mode of action

By definition, an immunostimulant is a "substance that enhances the immune system by interacting directly with cells of the system activating them" (Gannam & Schrock, 1999). The use of immunostimulants in fish diets has been accelerated in order to obtain natural substances which can heighten non-specific immunity. It is noteworthy that non-specific humoral and cellular responses showed differential activity to a given immunostimulant (Yano, 1996; Secombes, 1996). Moreover, the method of administration and the duration of administration are considered as important factors, and are variable with different compounds (Gannam & Schrock, 1999; Sakai, 1999). Conversely, some authors consider that the modes of action of immunostimulants are poorly understood (Galindo-Villegas & Hosokawa, 2004). Notwithstanding the contradictory views expressed by some workers, it would apparent that there are two main approaches for evaluating the efficacy of an immunostimulant:

(1) direct protection tests against fish pathogens

(2) measurement of the efficiency of innate cellular and humoral immune mechanisms, namely phagocytosis, respiratory burst, leucocyte proliferation and count, monocyte /lymphocyte/granulocyte count, lysozyme, complement, total protein, myloperoxidase, antiprotease and bacteriocidal activity.

Understanding how the fish immune system responds to foreign agents and how innate resistance can be selected by breeding processes to produce stocks of fish with higher disease resistance is the subject of ongoing research (Galindo-Villegas & Hosokawa, 2004). Shoemaker *et al.* (2001) published a schematic representation of the response of fish following an encounter with a pathogen (Fig. 4.1). Moreover, Gannam & Schrock (1999) stated that the non-specific immune system responds to general classes of non-self substances allowing quick mobilization of humoral and cellular parts of the immune system with key tissues or organs participating in the defense.

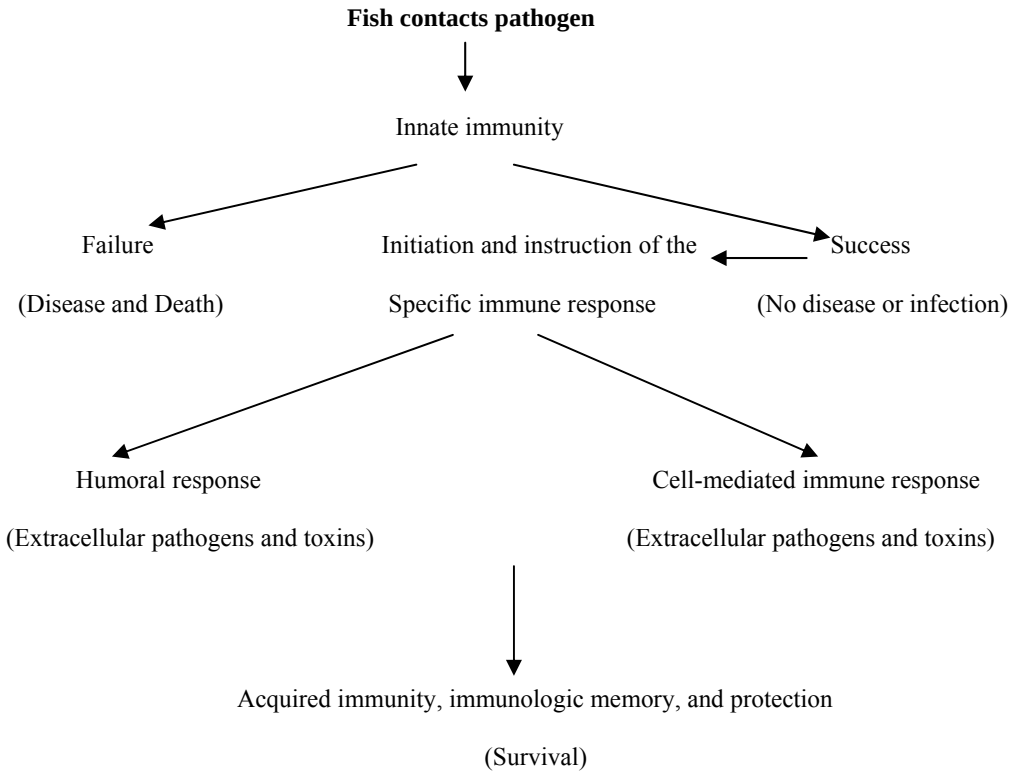


Fig. 4.1 Schematic representation of the response by fish following contact with a pathogen (after Shoemaker *et al.*, 2001).

One of the first methods for selection of candidate immunostimulants is a test of the resistance to a target pathogen, in this case *A. hydrophila,* after oral administration. Wassom & Kelly (1990) stated that the resistance level of fish to a particular disease can be determined from the survival rate after a bacterial infection. However, such findings of resistance activity need not be a good insurance for use *in vivo*, i.e. in field situations (Balcázar *et al*., 2006).

The results from this study have certainly reinforced the view that plant materials have a role in fish disease control strategies. All treatment doses led to protection against *A. hydrophila* infections in fish fed for 14 days, as compared with controls. Thus, the results parallel other work with rainbow trout, which involved feeding with ginger and garlic also for 14 days (Nya & Austin, 2009a, b). Also, rohu fed with garlic and mango showed resistance against *A. hydrophila* (Sahu *et al*., 2007a, b). Similarly, common carp fed with *Astragalus* (a leguminous herb) extract showed enhanced survival compared to control fish, also following challenge with *A. hydrophila* (Yin *et al.*, 2009).

It is interesting that the 1% dose of the plant material led to higher protection after challenge compared with the 0.5% dose, which is supported by other investigations. Thus, for example, feeding large yellow croaker with traditional Chinese medicine (*Astragalus* root and *Angelica* root) at 0.5, 1.0 and 1.5% led to the observation that the highest dose enabled better protection against challenge with *Vibrio alginolyticus* (Jian & Wu, 2003). Similarly, Kim *et al.* (1999) reported that juvenile rockfish fed with a diet supplemented with 0.1% aloe vera experienced higher mortalities than the group fed with a diet containing 0.5% aloe vera. Also, Rao *et al.* (2006) noticed an inverse relationship between the mortality rate after challenge with *A. hydrophila* and the percentage of prickly chaff-flower, *Achyranthes aspera* in rohu diet, i.e. the higher the percentage of *Achyranthes* in the diet, the less the level of mortality after challenge.

Interestingly, the 1% dose of lupin, mango and stinging nettle led to the highest resistance after 14 days, whereas the 2% level recorded the highest resistance after 21 days when challenged with *A. hydrophila*. In comparison, Ardó *et al*. (2008) recorded a reduction in the mortality of Nile tilapia after feeding for 28 days with Chinese herbs

(*Astragalus membranaceus* and *Lonicera japonica*) following challenge with *A. hydrophila*.

Haematological techniques, including erythrocyte count, haemoglobin concentration, haematocrit and leucocyte count, have provided valuable knowledge for fishery biologists in the evaluation of fish health (Blaxhall, 1972; Munkittrick & Leatherland, 1983) and in monitoring stress responses (Soivio & Oikari, 1976). Indeed, Garcia *et al.* (2007) suggested that haematological responses might be linked to either vitamin deficiency or hypervitaminosis. Some immunostimulants may exert a role also in organ function, notably to blood cell formation involving the thymus, spleen and bone marrow (Jeorg & lee, 1998). Of relevance, garlic has been reported to stimulate the immune system (Sahu *et al.,* 2007b; Ndong & Fall, 2007; Nya & Austin, 2009a), and led to an increase in haemoglobin content (Hb), haematocrit, and numbers of leucocytes and thrombocyte in Nile tilapia (Shalaby *et al.*, 2006). Also, there was an increase in leucocyte count in hybrid tilapia **(**Ndong & Fall, 2007). The results of this study were in agreement with previous research in which feeding with lupin, mango and stinging nettle led to an increase in erythrocyte and leucocyte count, haemoglobin level and haematocrit, compared to the controls. Similarly, haemoglobin percentage, and erythrocyte and leucocyte count were significantly higher in rohu fed for 20 and 40 days with 5 g and 10 g of mango kernel and garlic/kg of fish diet, compared with the controls (Sahu *et al.,* 2007a, b). Furthermore, Martins *et al*. (2002) recorded an increase in fish erythrocyte count, Hb content, Hct value, and leucocyte and thrombocyte numbers, after feeding with garlic. Certainly, common carp showed an increase in Hb, WBC and RBC after feeding with a herbal mixture prepared with the extract of 5 plants (elecampane, *Inula helenium*; coltsfoot, *Tussilago farfara*; black mustard, *Brassica nigra*; purple coneflower, *Echinacea purpurea* and greater celandine, *Chelidoniume mjus*) for 20 and 40 days. Decreases in erythrocyte count, haematocrit, and haemoglobin values could be an indicator of anaemia as a result of inhibition of erythropoiesis (Ates *et al.,* 2008). In addition, the study evaluated the effect of duration of administration on haematological parameters. However, there was not any change in fish haematological parameters after feeding for 14 days or 2 months, both of which led to an increase in values compared to the controls. Similarly, juvenile flounder fed for 2 months with a mixture of medicinal herbs (HM) (including medicated leaven, *medicata fermentata*; Japanese hawthorn fruit, *Crataegi fructus*; wormwood flower, *Artemisia capillaries*, and senkyu, *Cnidium*

officinale in the ratio of 2:2:1:1) showed increases in Hb and Hct values (Ji *et al*., 2007). In contrast the present study, pacu (*Piaractus mesopotamicus*), fed with dietary supplements of vitamins C and E did not lead to any change in total leucocyte, lymphocyte, monocyte and neutrophil counts (Garcia *et al*., 2007).

In teleosts, the anterior, head kidney have renal function only at early stage, it houses the site for haematopoiesis (Whyte, 2007). Moreover, the head kidney is the major site for antibody production, and the melanomacrophage accumulations of the parenchyma are able to retain antigens for long periods (Ronneseth *et al*., 2007). The head kidney contains a variety of cell types, including lymphocytes (T cell receptor genes), macrophages/monocytes, and granulocytes, all of which are able to induce immune responses to foreign material (Sorensen *et al.,* 1997; Schroder *et al.,* 1998; Stenvik *et al*., 2001; Wermenstam & Pilstrom, 2001). There are two types of lymphocytes, i.e. T-cells (thymus derived cells) and B-cells (bone marrow derived cells). T-cells are involved in cell-mediated immunity, which has an important role in killing viruses and intracellular pathogens, and secreting cytokines that initiate and regulate various immune responses (Secombes *et al*., 2005). B-cells produce and secrete antibodies (= immunogloblins = Igs), which are involved in specific humoral defense mechanisms (Yano, 1996). Macrophages and granulocytes are mobile phagocytic cells found in the blood and head kidney, which play an important role in inflammation as a direct response to microbial invasion (Iwama & Nakanishi, 1996). Neutrophil activity may be an indicator of the non-specific immune response (Weeks & Warinner, 1986), which exhibits increased production of oxygen radicals released during the oxidative burst process. These reactive species are capable of destroying the invading pathogens (Hassett & Cohen 1989; Lamas & Ellis, 1994).

The result of this research programme revealed an enhancement in differential leucocytes (lymphocyte, monocytes, and neutrophils) in the treatment groups, which were fed for 14 days, especially with the 1% and 2% doses. Similar findings were recorded by Nya & Austin (2009b) insofar as there were significant changes of rainbow trout lymphocyte, monocyte and neutrophil counts following feeding with ginger for 14 days. Furthermore, the total number of neutrophils of rainbow trout immersed in glucan was slightly higher after 10 days compared with the controls, which could be attributed to activation of the non-specific disease resistance mechanism (Gannam & Schrock,

1999). Moreover, feeding for 2 months with the same doses showed an enhancement in monocyte and neutrophil counts but not those of lymphocytes, which suggested that using those plant products in diets stimulated the innate immune system.

Phagocytosis is one of the main mediators of non-specific immunity to pathogens in fish. The principle cells involved in this defence mechanism are phagocytic cells; i.e. neutrophils, monocytes and macrophages, which are able to engulf invading pathogen and then digest the cellular debris as well as stimulating lymphocytes and other immune cells to respond to the pathogen (Rao *et al*., 2006). Also, phagocytic cells play a vital role in promoting synthesis of antibody (Gan, 1998). An increase in phagocytic activity by immunostimulants has been documented by many authors (e.g. Gannam & Schrock, 1999; Dügenci *et al.,* 2003; Yin *et al*., 2006; Ardó *et al*., 2008). However, the ideal time and dose of immunostimulants for enhancement of immunity is variable. Jeney & Anderson (1993a) recorded an increase in phagocytic activity in rainbow trout after exposure to glucan for 1 day, with the activity peaking at 3-4 days. Also, garlic led to a significant increase in activity after 4 weeks, compared with 2 weeks for hybrid tilapia (Ndong & Fall, 2007). In this study, the highest phagocytic activity was recorded in the groups which were fed with 1% doses of lupin, mango and stinging nettle, followed by the 2% and 0.5% doses, respectively. This finding concurs with those of Yuan *et al.* (2007), who observed increases in the phagocytic activity of common carp after feeding with 1% of traditional Chinese medicines (TCM), more so than the 0.5% dose. Also, rainbow trout fed with 1% of stinging nettle and garlic recorded higher phagocytic activity than in the 0.1% dose, which was greater that the controls (Dügenci *et al*., 2003). Conversely**,** Yin *et al*. (2006) found that tilapia fed with *Scutellaria* extract at higher doses (0.5 and 1.0%) had a reduced function in the phagocytic cells whereas when fish were fed with a lower dose, i.e. 0.1%, there was not any effect on phagocytic activities. Certainly, the enhancement in phagocytic activity of macrophages in treatment groups, especially those receiving the 1% and 2% doses, could be responsible for the lower mortality recorded after challenge with *A. hydrophila*.

Several forms of reactive oxygen intermediates are produced during phagocytosis. These include superoxide anion (O_2^-), hydroxyl radical ($OH\cdot$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Munoz *et al*., 2000), which are considered as toxic for bacterial fish pathogens (Hardie *et al*., 1996; Itou *et al*., 1996). Superoxide anion is the

first product to be released from the respiratory burst; therefore the measurement of O_2^- has been accepted as an accurate method of measuring this activity (Secombes, 1990; Roch, 1999). There are two main methods to measure O_2^-: firstly, the reduction of ferricytochrome$_c$ to determine extracellular O_2^-, and secondly, the reduction of nitroblue tetrazolium (NBT) to determine intracellular O_2^- (Dügenci *et al*., 2003). Generally, all the treatment doses, especially the 1% doses, in this study led to increased respiratory burst activity compared to the controls. The high phagocytic and respiratory burst activities which were recorded in fish that received the 1% doses could be attributed to the fact the respiratory burst activity could be correlated with increased bacterial pathogen killing ability of the phagocytes during phagocytosis (Sharp & Secombes, 1993). Of relevance, Dügenci *et al*. (2003) reported increased extracelluar oxidative radical production and phagocytic activity in rainbow trout administered with 1% and 0.1% ginger, mistletoe and nettle. Similarly to the present study, Jian & Wu (2004; 2003**)** recorded significantly increased respiratory burst activity in Jian carp and yellow croaker fed for 20 days with 1% and 1.5% of TCM (mixture of Astragalus; *Astragalus membranaceus* and *A. sinensis* extracts). In rohu, feeding with three doses of mango (1%, 5% and 10%) led to increases in superoxide anion production (Sahu *et al.,* 2007a). Again, it should be emphasised that a high dose does not necessarily enhance or indeed inhibit the immune response (Robertsen *et al*., 1994; Kajita *et al*., 1990). For example, Robertsen *et al.* (1994) recorded maximal respiratory burst activity in glucan-treated macrophages after exposure to concentrations of 0.1–1 µg/ml, whereas 10 µg/ml did not lead to any demonstrable effect. In contrast to the present study, Xue *et al.* (2008) recorded a rise in respiratory burst activity of abalone fed with increasing proportions of TCM. Similarly Ndong & Fall (2007) reported significantly increased respiratory burst activity in hybrid tilapia fed with 0.5% garlic more so than a 1% dose for 4 weeks. Furthermore, feeding rohu with *Achyranthes* (0.5%) followed by challenge with *A. hydrophila* revealed elevated superoxide anion production compared to the controls (Rao *et al*., 2006).

Lysozyme is an important parameter in the immune defence of both invertebrates and vertebrates. Lysozyme is a cationic enzyme found in cutaneous mucus, peripheral blood and certain tissues rich in leucocytes that break β (1-4) glycosidic acids and N-acetyl glucosamine in the peptidoglycan of bacterial cell walls (Wang *et al.,* 2010). Lysozyme attacks mainly Gram-positive bacteria and some Gram-negative organisms in

conjunction with complement (Alexander & Ingram, 1992; Jollès & Jollès, 1984). In fish, lysozyme is recognised to be an opsonin, and activates the complement system and phagocytes (Grinde, 1989). Therefore, lysozyme plays an important role in the host defense mechanisms against infectious diseases (Lundblad *et al.,* 1979; Lindsay, 1986; Lie *et al.,* 1989). The results of this study revealed a significant increase in lysozyme activity in all treatment groups after feeding for 14 days, especially in animals receiving the 1% and 2% doses. Yet, feeding for 7 days did not reveal much difference in activity relative to the controls. Similarly to this study, feeding rohu for 14 days with 0.5% prickly chaff-flower led to enhancement in lysozyme activity (Rao *et al*., 2006). Also, feeding with 1, 5 and 10 g mango kernel/kg led to increased activity in rohu (Sahu *et al*., 2007a). In contrast to this study, Ardó *et al.* (2008) recorded elevation in lysozyme levels of Nile tilapia fed for 7 days with Chinese herbs, *A. membranaceus* and *Lonicera japonica*, alone or mixed. Indeed several authors recorded differences in lysozyme activity after administration of different doses of immunostimulants for various durations. For example, garlic fed at 0.5% for 2 and 4-weeks was attributed with increasing lysozyme activity in hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*) (Ndong & Fall 2007), and in rohu fed with 0.1, 0.5 and 1.0% garlic for 60 days (Sahu *et al*. 2007b). Other examples involved TCM as an immunostimulant, which led to elevated lysozyme activity after feeding Jian carp for 20, 25 and 30 days (Jian & Wu, 2004). Furthermore, feeding Nile tilapia for 1 week with 0.1 and 0.5% *Astragalus radix* enhanced lysozyme activity (Yin *et al*., 2006). Similarly, feeding Chinese herbs, *A. radix* and *Ganoderma lucidum,* induced enhanced lysozyme activity in common carp (Yin *et al.,* 2009). Moreover, an aqueous extract of *Eclipta alba* leaf incorporated into the diet and fed for 2 and 3 weeks led to a significant increase in the lysozyme activity of common tilapia, *Oreochromis mossambicus* (Christybapita *et al.,* 2007).

Complement is a common protective system in vertebrates, containing ~35 soluble glycoproteins and membrane-bound proteins present in blood and other body fluids, which interact sequentially with each other leading to cleavage of some complement components into active fragments (e.g. C3 is cleaved to C3a and C3b) contributing to activation of the next complement component leading to lysis or protection against a variety of micro-organisms (Lydyard *et al*., 2004). The complement systems has three pathways of activation:

The classical complement pathway (CCP)

The alternative complement pathway (ACP)

The lectin complement pathway (LCP).

With the exception of jawless fishes, all pathways have been identified in fish (Fujii, *et al.,* 1992; Nonaka, 1994). ACP is directly activated by LPS in the cell walls of bacteria, resulting in lysis (Ellis, 2001). The first pathway to be discovered was CCP, which is triggered by the binding of antibody to a cell surface (Claire *et al.*, 2002). LCP is initiated by binding of a complex protein consisting of mannose-binding lectin and serine proteases and mannose-binding lectin associated proteases 1 and 2 to mannans on bacterial cell surfaces (Galindo-Villegas & Hosokawa, 2004). In fish, the complement system plays important roles for bacteriocidal activity in serum and mucus (Ellis, 2001**;** Holland & Lambris, 2002) and as an opsonin, encouraging phagocytosis by reacting with specific binding sites on the phagocyte surface (Leiro *et al.,* 1996; Jenkins & Ourth, 1993). The alternative complement pathway may be activated by immunostimulants (Jeney & Anderson, 1993b; Engstad *et al.,* 1992). The results of this study showed enhancement in complement activity in fish fed with lupin, mango and stinging nettle especially those receiving the 1% and 2% doses. In particular, the highest activity was seen after feeding for 14 days with the 2% dose. Similarly, feeding Jian carp and yellow croaker with 1% and 1.5% TCM (mixture of astragalus root and angelica root) for 20 and 30 days led to significantly increased complement activity (Jian & Wu, 2004; 2003). Although aqueous *E. alba* leaf extract resulted in significantly increased complement in tilapia after 1 and 2 weeks, the activity declined at 3 weeks (Christybapita *et al.,* 2007). Several authors reported an increase in complement activity following administration of immunostimulants. For example, sea bass fed diet containing sodium alginate for 15 days showed significant increases in complement activity (Bagni *et al.,* 2005). Similarly, feeding brown-marbled grouper, *Epinephelus fuscoguttatus,* with a diet containing sodium alginate and k-carrageenan over 2–8 weeks led to significant increases in alternative complement activity (Cheng *et al.,* 2008). In addition vitamins have been used to successfully induce immunity in fish. Thus, dietary vitamins C and E resulted in heightened levels of complement as well as phagocytic activities of gilthead sea bream (Ortuno *et al.*, 2001). Also, Ai *et al.* (2007) reported significantly higher serum lysozyme, phagocytosis and respiratory burst activity in head kidney with increasing dietary vitamin C levels in yellow croaker. Conversely, in Atlantic salmon there were not any indications of an effect of dietary vitamin A on

complement activity (Thompson *et al.,* 1993). Furthermore, Wahli *et al.* (1998) reported the lack of any strong influence on complement activity of rainbow trout after administering food supplemented with vitamin C.

Various humoral factors involved in innate and/or adaptive immunities are elevated in the serum to protect the host from infection (Rao *et al.,* 2006). Serum bactericidal activity is a mechanism noted for the killing and clearing of pathogenic organisms in fish (Ellis, 2001). The results of this study revealed higher serum bactericidal activity especially in higher doses, i.e. 1 and 2%, of the plant products with the highest activity recorded after 21 days. The results revealed that the dietary supplements helped to heighten the humoral immune elements in the serum. Similarly, serum bactericidal activity was significantly increased in common carp following the use of greater amounts herbal extracts (mixture of elecampane inula *Inula helenium;* coltsfoot, *Tussilago farfara;* mustard, *Brassica nigra;* purple coneflower, *Echinacea purpurea* and greater celandine, *Chelidoniume majus*) in diets (Mohamad & Abasali, 2010). This is in agreement with the work of Rao *et al.* (2006), who noticed greater serum bactericidal activity in rohu administered with dietary supplements comprising prickly chaff-flower seed at 0.01, 0.1 and 0.5%. Furthermore, the activity increased gradually with higher proportions of the seed. Similar observations were made by Punitha *et al.* (2008), who recorded significant increases in the serum bactericidal activity in grouper juveniles administered with diets containing different doses of a herbal mixture (Bermuda grass, *Cynodon dactylon;* long pepper, *Piper longum;* Stonebreaker, *Phyllanthus niruri;* coat buttons, *Tridax procumbens* and ginger, *Zingiber officinalis*). However, it is unclear why the immune response in some species is enhanced after administering immunostimulants/plant products for comparatively short periods whereas the process takes much longer in other taxa. For example, enhancement in bactericidal activity was recorded in rainbow trout fed with garlic for 14 days (Nya & Austin *et al.,* 2009a). Conversely, in rohu, dietary supplements with mango and garlic resulted in increased serum bactericidal activity after 20 and 40 days, with the maximum activity taking 60 days (Sahu *et al.,* 2007a, b). Also, greasy grouper had higher serum bacteriocidal activity after administering different concentrations of tulsi, *Ocimum sanctum* and ashwagandha, *Withania somnifera,* extract whereas the extract of nutmeg, *Myristica fragrans,* did not result in any enhancement (Sivarama *et al*., 2004). Conversely to this study, Thompson *et al*. (1993) reported a lack of any effect on

phagocytosis, respiratory burst and bacteriocidal activity when Atlantic salmon were fed with vitamin A.

Neutrophils contain myeloperoxidase (MPO) in their cytoplasmic granules (Afonso *et al.,* 1997; Rodriguez *et al*., 2003). MPO is an important enzyme with microbiocidal properties, which utilises one of the oxidative radicals (H_2O_2) to produce hypochlorous acid (Dalmo *et al.* 1997). This process is believed to be important in killing micro-organisms (Johnston, 1978). This study revealed an increase in MPO content in the treatment groups compared to the control, especially after feeding with 1 and 2% doses for 1 and 2 weeks. In agreement with this study, MPO activity increased significantly in common tilapia fed with diets supplemented with different levels of aqueous extract of false daisy, *Eclipta alba,* leaf for 1 week, whereas feeding for 2 or 3 weeks did not result in any significant increase (Christybapita *et al.,* 2007).

Other immunostimulants have been reported to lead to enhancement of MPO activity. For example, the MPO content was enhanced in sea bream after feeding for 2 or 4 weeks with 300 mg of vitamin A/kg fish diet (Cuesta *et al.,* 2002). Also, carp injected with levamisole showed enhanced phagocytic activity and MPO activity in neutrophils as well as elevation in serum lysozyme levels (Siwicki, 1987; 1989). Similarly, Anderson *et al.* (1995) recorded an increase in immunological parameters of rainbow trout, including NBT, potential killing activity and MPO levels, after injection or immersion with/in chitosan. Conversely, Asian catfish, *Clarias batrachus,* injected with cyclophosphamide developed a significant decline in respiratory burst and MPO (Kumari & Sahoo, 2005). Probiotics have also led to enhancement in serum MPO content and the natural haemolytic complement activity, especially after administration for 3 and 4 weeks (Díaz-Rosales *et al*., 2006).

Antiproteases (= protease inhibitors) play vital roles in the defence of various organisms by regulating and inhibiting the activities of potentially destructive proteases. Generally, antiproteases inhibit the action of proteases either by binding to their active sites or by 'trapping' the protease to prevent protein hydrolysis (Laskowski & Kato, 1980) and, therefore, restrict the ability of bacteria to invade and to grow in fish (Ellis, 2001). Also, proteases may be involved with the activation of haemolysin (Howard & Buckley, 1985).

All doses, especially the 1% dose, used in these experiments increased antiprotease activity after feeding for 14 days. These findings were in general agreement with those of Christybapita *et al.* (2007), who noticed significantly enhanced levels of serum antiprotease activity in tilapia fed with false daisy, *E. alba,* aqueous extract-incorporated diet for 2 or 3 weeks. Also, Nya and Austin (2009a) recorded significantly higher serum anti-protease activity in rainbow trout fed with ginger for 14 days. These workers highlighted the increased activity commensurate with the higher doses. In yet further examples, dietary supplementation with prickly chaff-flower seed led to maximum levels of serum antiproteases in catla, *Catla catla,* on day 21, and decreased by day 28 (Rao & Chakrabarti, 2005). However in rohu, the maximum level was recorded during the first week and thereafter the levels gradually decreased to the fourth week (Rao & Chakrabarti, 2004). Other immunostimulants have led to similar results. For example, rainbow trout fed with a mixture from astaxanthin and vitamin A supplemented diet for 4 months developed significantly enhanced levels of serum antiprotease activity (Thompson *et al.*, 1995). A similar observation was made using Atlantic salmon when fed with vitamin A (Thompson *et al.*, 1994).

Fish protein possesses a high variability in its composition, which largely reflects the functional status of the organism (Afanasjev & Afanasjev, 2001). Serum proteins have a variety of functions, starting with regulation of the water balance in fish (Wedemeyer & Yasutake, 1977), and enable protective effects (Afanasjev & Afanasjev, 2001) through the role of acute phase proteins in limiting the dispersal of infectious agents by repairing tissue damage and killing micro-organisms (Larsen *et al.,* 2001; Gerwich *et al.,* 2002). In common with the present study, the enhancement of total protein by using immunostimulants/plant products has been widely observed in fish. In particular, Dügenci *et al*. (2003) recorded enhanced total protein levels in rainbow trout fed with 0.1% and 1% ginger, mistletoe and stinging nettle. Similarly, the highest serum protein level was recorded in Nile tilapia fed for 2 and 3 weeks with 0.1% yellow leader, *A. membranaceus,* and Japanese honeysuckle, *Lonicera japonica* (Ardó *et al*., 2008). Moreover, the effective feeding period to achieve the highest protein level was reported to be 14 days in the case of the 1% dose, with a gradual decline at 21 and 28 days. In our study, the highest protein level in the groups fed with the 2% dose was achieved after 7 and 14 days, with a decline thereafter. Possibly, this is due to the effect of dosage

and the duration of administration (Sakai, 1999). With the exception of mango, higher serum total protein levels were recorded in fish fed for two months with the 1% dose, more so than 2%. Also, Mohamad & Abasali (2010) recorded increases in total protein, albumin and globulin levels in common carp fed for 20, 40 and 60 days with different doses of extracts of a herbal mixture. These workers suggested that high concentrations of total protein, albumin and globulin levels in fish serum were likely to be as a result of enhancement of the non-specific immune response.

Proteins include albumin and globulin; the former of which is synthesized in the liver (Sandnes *et al.,* 1988). Globulin is made up of fractions of α1, α2, β, and γ globulins, which are considered as the source of almost all the immunologically active protein in the blood (Jha *et al.,* 2007). Generally, increases in the levels of serum protein, albumin and globulin in fish is thought to be associated with a stronger innate response (Wiegertjes *et al.,* 1996). Although albumin did not increase in most of the treatment groups in the present study, globulin responded similarly to total protein, which certainly increased. It is apparent that many investigators have recorded increases in total protein, albumin and globulin in fish serum after administration of herbal compounds/immunostimulants. For example, feeding diet supplemented with 0.1, 0.5 and 1% garlic and 1, 5 and 10% mango kernel led to higher levels of total protein, globulin and albumin in the serum of rohu (Sahu *et al.,* 2007a,b). Furthermore, Yuan *et al*. (2007) recorded significant increases in total protein, albumin and globulin in common carp after administration of diets containing 0.5 and 1% of a herbal mixture of Chinese medicine comprising yellow leader (*A. membranaceus),* Hoshouwu *(Polygonium multiflorum),* woad *(Isatis tinctoria)* and liquorice *(Glycyrrhiza glabra)* for 30 days. Similarly to the present study, the maximum level of total protein and globulin in rohu serum was recorded after feeding for 14 days with 0.5% prickly chaff-flower, with those levels gradually decreasing at 21 and 28 days (Rao *et al.,* 2006). Furthermore, juvenile greasy groupers showed significant increases in serum globulin level after feeding with100 and 200 mg/kg of tulsi and Ashwagandha (*W. somnifera)* for 12 weeks (Sivaram *et al.,* 2004).

Of relevance to the current study, experiments have been conducted previously to evaluate the efficacy of herbs to treat bacterial diseases and enhance the immune system. For example, Harikrishnan *et al*. (2003) infected common carp with *A.*

hydrophila. After the disease signs were noticed on the 7th day, the fish were dipped into an aqueous leaf extract of neem, *Azadirachta indica,* daily for 30 days until the lesions healed completely. The haematological and biochemical parameters of the infected and control fish were monitored on day 10, 20 and 30, with the results revealing increases in RBC, Hb, Hct and total protein. The authors considered that soluble and particulate components of the aqueous leaf extract were involved in the development of a protective immune response.

Since the head kidney is rich in several types of immune cells which play vital roles in the defence mechanisms against foreign molecules and are involved in the development of the adaptive immune response of fish, it was decided to examine the production of immunomodulatory cytokines. Generally, fish produce a number of cytokine-like soluble products, which exert a regulatory or enhancing role in the immune system. Most of the cytokines have been identified in biological assays on the basis of their functional similarity to mammalian cytokines (Plouffe *et al.,* 2006), and some have been detected though their cross-reactivity [with mammalian cytokines] (Secombes *et al.,* 2001). In brief, cytokines are subdivided into families, including interleukins (IL), transforming growth factors (TGF), interferons (IFN), and chemokines. IL-1β is one of the early response proinflammatory cytokines, which stimulates immune responses by activating lymphocytes or by inducing the release of other cytokines that are able to activate macrophages, NK cells and lymphocytes (Low *et al.*, 2003). For example, head kidney leucocytes from trout cultured with LPS showed an increase in IL-1β expression (Secombes *et al.,* 1999). Furthermore, a trout IL-1β derived peptide has been shown to stimulate macrophages by enhancing phagocytosis and bacteriocidal activity against *A. salmonicida in vitro* (Peddie *et al.,* 2002). IL-8 is produced in response to stimulation by proinflammatory cytokines or bacterial LPS (Vaddi *et al.,* 1997; Chen *et al.,* 2005). Also, IL-8 can attract neutrophils, T-lymphocytes and basophils to inflammatory sites (Mukaida *et al.,* 1998). In particular, IL-8 was detected in head kidney and spleen of catfish. Moreover the expression was upregulated 3-5 fold in channel catfish and blue catfish after infection with *Edwardsiella ictaluri* (Chen *et al.,* 2005). TGF-ß1 is involved in the wound repair processes and in starting inflammatory reactions, and then in their resolution and haematopoiesis (Lawrence, 1996). This cytokine induces active immune tolerance in mucosal and peripheral tissues and exerts profound effects on immune cells, including lymphocytes, macrophages and dendritic cells (Letterio &

Roberts, 1998). Generally, IL-1β and TGF-ß have multiple isoforms (Pleguezuelos *et al.,* 2000; Laing *et al.,* 2000), but single isoforms for each was used in this study.

Many investigations have focused on identifying cytokines and other immune related genes in rainbow trout, and specifically the role within the salmonid immune system (Secombes *et al.,* 2001; Wang *et al.,* 2002; Laing *et al.,* 2002; Zou *et al.,* 2003). Komatsu *et al.* (2009) demonstrated that rainbow trout could produce several kinds of cytokines in response to bacteria. Also, workers showed that the expression of the IL-1ß and TNF-α genes in intestinal epithelial cells increased in response to pathogenic *A. salmonicida,* but not in response to non pathogenic *E. coli*. However, formalin-killed cells of *A. salmonicida* failed to increase expression. Conversely in humans, cells secrete certain interleukins in response to both pathogenic and non-pathogenic bacteria, via the cytokine response patterns of epithelial cells (Hosoi *et al.,* 2003). The current study aimed at detecting up/down regulation of three types of cytokine; IL-1ß, IL-8 and TGF-ß1, in rainbow trout fed for 2 months with dietary 1% and 2% lupin, mango and stinging nettle and challenged with *A. hydrophila,* in order to measure the expression of cytokines in response to pathogen. The outcome was that the data showed upregulation of genes in the group fed with 1% but not really 2% lupin. With mango, only the 1% dose recorded significant upregulation in the TGF-ß1 gene. However, all the doses of stinging nettle upregulated genes, significantly. Therefore, it was concluded that both lupin and stinging nettle stimulated the expression of IL-1ß, IL-8 and TGF-ß1 in head kidney. Similarly, Yuan *et al*. (2008) studied the expression of the immune response genes in head kidney, gill and spleen of common carp injected with *Astragalus* polysaccharide. Here, the result showed increases in the IL-1ß mRNA level of the head kidney, but no significant change in the gill and spleen. Also, an aqueous extract of Lingzhi/Reishi mushroom enhanced phagocytosis by macrophages in mice immunosuppressed by cyclophosphamide, and influenced the gene expression of cytokines (Wang *et al*. 1997).

In fish, immunostimulants have been demonstrated to stimulate the innate and adaptive immune system and increase the resistance against pathogens. Ergosan has been shown to exert a positive effect on liver cytokine (IL-1ß, IL-8 and TNF-α) gene expression in rainbow trout leading to a significantly higher expression, thus indicating a role in stimulating the innate immune response (Gioacchini *et al.,* 2008). Furthermore,

analysis of IL-1β and TNF-α gene expressions in common carp, orally treated with the blue green algae spirulina (*Spirulina plantensis*) showed upregulation, although there was down-regulation in IL-10 gene (Watanuki *et al.,* 2006). Similarly, Chansue *et al.* (2000) recorded an increase proteins reactive with antibodies to human IL-Iß, IL-10, IL-12 and TNF-α in Nile tilapia after oral administration of ß-1,3-glucan.

Probiotics have been reported to effect cytokine expression in fish. For example, Panigrahia *et al.* (2007) studied the expression of IL-1ß1, IL-1ß2 and TGF-ß1 in spleen and head kidney of rainbow trout following dietary administration of probiotics for 45 days. The results showed significantly higher IL-1ß1 expression in spleen and kidney compared to the control, although IL-1ß2 was not expressed or affected. The TGF-ß gene was upregulated in spleen and kidney. Separately, Kim & Austin (2006) evaluated the expression of cytokine genes in head kidney leucocytes and gut cells isolated from rainbow trout after co-culturing with live probiotic, i.e. *Carnobacterium maltaromaticum* B26 and *C. divergens* B33, and/or a pathogen, *Y. ruckeri*. The results indicated stimulation of the expression of IL-1ß, IL-8, TNF- α and TGF- ß (only in co-culture with *Y. ruckeri*) in head kidney leucocytes. However, significant differences were not found in gut cell co-culture except for co-culture with *Y. ruckeri* for 12 h (Kim & Austin, 2006).

4.3 Physiological and biochemical effect of long term feeding

The present study was aimed at investigating the effect of long term feeding with lupin, mango and stinging nettle on physiological parameters. Moreover, since some of the plants are rich in anti-nutritional factors, the interaction between diet composition and the digestibility in fish could be relevant. However, it was argued that the normal physiological-biochemical parameters could differ during different seasons, reproductive periods and under unnatural rearing such as in aquaria (Martem'yanov, 2001).

Electrolytes are required for normal life processes in fish. Some of them, for example, manganese (Mg), Zinc (Zn) and iron (Fe), are obtained from the diet or from the surrounding water. Generally, electrolytes carry out several homeostatic functions, such as bone and teeth formation, polarization of membranes, integration of enzymatic systems, energy storage, acid-base balance, clotting and respiration (Watanabe *et al*.,

1997; Coppo, 2001). Moreover, mineral deficiencies may cause biochemical, structural and functional pathologies, which depend on several factors principally the duration and degree of mineral deprivation. Sugiura *et al.* (1998) suggested that the availability of minerals increased when diets became acidified, and decreased when basic components were added to the diets.

Fe is the most important element which plays an active part in oxidation/reduction and electron transport associated with respiration (Watanabe *et al*., 1997). Therefore, it helps in the killing activity of neutrophils and lymphatic tissues and respiratory tract mucosa (Jamroz, 2005). Fe deficiency causes anaemia, and makes the animal more susceptible to disease. The results of the present study showed an increase in Fe level in the treatment groups, and this could be attributed to the same observation of the efficiency of treatment to enhance immunity.

Mg is necessary for functioning of brain, for lipid and carbohydrate metabolism (Watanabe *et al*., 1997) and for enhancement of the immune system, which activates natural killer-cells and the activity of macrophages (Jamroz, 2005).

Zn in involved in various metabolic pathways, namely influencing thymic functions and their hormones, increasing pancreatic enzymatic activity, and stimulating helper lymphocytes T_H1. Also, Zn is one of the trace elements that increases WBC production and improves the release of antibodies, decreases infections, shows antimicrobial activity, and improves the absorption of water, electrolytes (Na), glucose and other saccharides, amino acids and lipids (Jamroz, 2005). According to Gatlin & Wilson (1983), a low level of Zn in fish diets may reduce appetite leading to low growth, low bone Zn and Ca levels, and serum Zn concentration. This may explain the reason for the decrease in growth rate in control fish coinciding with a decrease in Zn levels in fish plasma.

Ca is very important in fish where it plays a vital role in maintenance of acid-base equilibrium and cell membrane formation, nerve transmissions, and activation of enzyme activity and bone composition (Lall, 2002). Na and K are ions which affect osmotic pressure and stress factors. Furthermore, a decrease in Na concentration and an increase of K concentration in the plasma are observed at the initial phase of acute stress

(Martem'yanov, 1983; Martem'yanov & Zaprudnova, 1982). Higher K levels were recorded in control groups in the present study, but the Na level did not record the lowest value. Although the electrolyte data in this study was variable, it was still within the normal range of rainbow trout as documented by Hille (1982). Furthermore, dietary supplementation of vitamin C and D reduced in Na, Mg and Ca levels in rainbow trout, although Fe was higher in the treatment groups (Sugiura *et al.*, 1998).

The liver exerts an important role in lipid metabolism, including fatty acid synthesis and degradation through enzyme regulations. Moreover, it is a sensitive organ reflecting dietary lipid change in fish (Kiessling & Kiessling, 1993; Henderson, 1996). GOT **(**glutamic oxaloacetic transaminase**)** and GPT (glutamic pyruvic transaminase) enzymes are two liver enzymes which function in the transference of amino groups from alph-amino to alph-keto acids. Large amounts of GOT and GPT are released into blood during liver cell damage. Therefore, detection of them in serum above the normal range indicates liver cell damage (Soltan *et al*., 2008). Soltan *et al*. (2008**)** recorded increased levels of GOT and GPT in Nile tilapia following the feeding with increased amounts of plant protein mixture (PPM). These workers attributed abnormalities in liver function with anti-nutritional and toxic factors present in PPM. Similarly the present study revealed increases in serum GOT and GPT in rainbow trout fed with 1 and 2% lupin, mango and stinging nettle. However, the levels were within the normal range for rainbow trout (Hille, 1982; Sandnes *et al.,* 1988). Similarly, rohu fed with dietary vitamin C at 500, 1000 and 1500 mg kg^{-1} showed no effect on GOT and GPT levels (Tewary & Patra, 2008). Yet in rats, there was a decrease in GPT and GOT after feeding with diets supplemented with ginger (Egwurugwu *et al.,* 2007).

Other function of kidney is ultrafiltration, in which water and salt concentrations within the fish body are regulated. This permits certain fish species to exist in freshwater or saltwater, and in some cases (e.g. snook, tarpon and salmon) in both. Creatinine is formed by spontaneous cyclization of creatine. In fish, creatinine levels are always low and appear to be unaffected by stress (Wells *et al*., 1988). Uric acid is generally converted to urea for excretion. A reduction in food intake over a period of several weeks can reduce urea and hence the ability for hyperosmoregulation (Hoar *et al.,* 1992). Moreover, some plants are used to reduce kidney function, for example, stinging nettle has been used in one month treatment regimes to reduce serum creatinine and urea levels in patients (Treasure, 2003). Also, replacement of soybean meal in rabbit

with radish, rocket and black cumin meal (at a level of 50%, respectively) recorded improvement in GOT, GPT and urea, whereas there were not any changes for creatinine (El-Nattat & El-Kady, 2007). Therefore, a thrust of the present study was to check the long term effect of using plants in fish nutrition. Here, the results showed an increase in urea and creatinine in controls compared to treatment groups. Of relevance, Zaki *et al.* (2008) associated the elevation of serum creatinine and urea in fish with Na and K. This agrees with the present study which showed increases in Na and K in controls, although there was not any significant difference between controls and treatment groups, except in creatinine levels.

Generally, glucose level increases in infected or stressed animals to ward off infection or the effects of stress (Citarasu *et al*., 2006). Similar conclusions were made by Mohamad & Abasali (2010), who observed an inverse relationship between glucose level and concentration of extract of herbal mixtures fed to common carp. These workers attributed this observation to the capability of herbal extracts to reduce the effects of stressors. Furthermore, rohu fed with garlic and mango kernel for 60 days showed reduction in glucose levels compared to controls, except in the group fed with 5% mango (Sahu *et al*., 2007a, b). This observation may be attributed to the hypoglycaemic activity of some plant extracts to increase the level of serum insulin (Eskander & Won Jon, 1995) and to the enhancement of peripheral metabolism of glucose (Skim *et al.,* 1999). Furthermore, Ji *et al*. (2007) reported low blood glucose levels and plasma GOT in juvenile Japanese flounder fed with herbal mixtures. The observations were attributed to the activation of glycogen synthesis and healthy hepatic function (Ji *et al.,* 2007). Conversely in this study, feeding rainbow trout with 1% and 2% lupin, mango and stinging nettle for 2 months led to increases in glucose level in all the treatment groups, compared to the controls. Indeed, the increase in glucose was accompanied with greater levels of GOP and GPT, even allowing for the latter two being in the normal activity range, although there may well have been a sign of problems in liver function. Similar to the present study, the glucose level increased in common carp treated with aqueous neem, *A. indica,* leaf extract after challenge with *A. hydrophila* (Harikrishnan *et al.*, 2003).

4.4 Effect of long term feeding on growth rate

The nutritional status is important in determining the ability of fish to resist disease. Therefore, there is a clear need for a proper diet to improve health and to prevent outbreaks of disease. Several authors have dealt with aspects of nutrition and the immune response in fish (Blazer, 1992; Lall & Olivier, 1993). Of relevance, a significant positive correlation has been found between increasing disease resistance and growth rate and survival (Fjalestad *et al.,* 1993). For example, Gannam & Schrock (1999) related an increase in growth rate with immunostimulation and general fish condition resulting from administration of glucan. Conversely, partial or total replacement of fish meal by a mixture of plant protein sources (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin) in juvenile gilthead sea bream resulted in enhancement of immune parameters, namely complement activity, respiratory burst and MPO values. However, there was a reduction in growth performance (Sitjà-Bobadillaa *et al.,* 2005). In addition, utilization of dietary nutrients can be reflected in the metabolic profile of fish tissues as metabolic efficiency determines growth characteristics. Certainly, there is an interest to replace fish protein in fish diets with cheaper more sustainable sources, such as plant protein (Gouveia *et al.,* 1993; Bangoula *et al.,* 1993; Glencross *et al.,* 2004). However, the total replacement of fish meal with plant protein has not been recommended by some authors as there was a decrease in growth performance with increasing incorporation of plant meal (Fontainhas-fernandes *et al.,* 1999; Glencross *et al*., 2004). These workers attributed the higher levels of crude fibre and anti-nutritional compounds in feeds with an effect on protein digestibility leading to adverse physiological effects and reduction in growth (Olli *et al.*, 1994; Vielma *et al.,* 2000; Ali *et al.,* 2003). However, replacing half of the fish meal with an equivalent amount of plant material may be a good compromise. In this connection, Fournier *et al.* (2004) found that replacement of fish meal by plant meal, i.e. lupin, corn gluten and wheat gluten meal, in the diets of juvenile turbot (*Psetta maxima*) by up to 50% did not significantly affect growth rate, whereas a 75 or even 100% replacement significantly reduced growth. Similarly, Bilgin *et al.* (2007) observed that hazelnut meal could replace 20% and 30% of dietary soybean meal without growth suppressing effects on specific growth rate, relative growth rate, feed efficiency, and survival. The present study recorded remarkable increases in weight gain and SGF in treatment groups compared with the controls. Certainly, Jian & Wu (2004) reported an increase in body weight of Jain carp after feeding with a TCM

formulation of Astragalus root (*Radix astragalin*) and Chinese angelica root (R. *angelicae sinensis*) at a ratio of 5:1. Also, the weight gain was significantly improved in Japanese flounder, *Paralichthys olivaceus*, fed with 0.5 % of a herbal mixture (Ji *et al.,* 2007). Similarly, replacing fish protein with a mixture of plant protein source (soya bean, alfalfa meal and corn gluten) at 50% replacement led to a significant increase in SGF and final body weight in Nile tilapia. However replacing 100% of the fish protein did not result in much difference compared to the controls (González-Félix *et al.,* 2009). Similarly, Nya & Austin (2009b) recorded increases in SGR and FCR in rainbow trout fed with different doses of ginger. In contrast, body weight gain was not affected in juvenile hybrid tilapia fed diets supplemented with garlic at concentrations of 0.5% and 1% over 4 weeks (Ndong & Fall, 2007). Similarly, growth in rainbow trout fed with plant meal diet was significantly less than fish fed the fishmeal diet (Pierce *et al.,* 2008). Yet, Rao *et al.* (2006) reported elevated specific growth rates in rohu after feeding with prickly chaff-flower. Rohu fed with mango kernel showed an increase in SGR and FCF, although there was not any significant difference between each treatment and the controls (Sahu *et al.,* 2007b). Interestingly, Olvera-Novoa *et al.* (1990) reported that weight gain, SGR, feed intake and nitrogen deposition in tilapia were best with low levels, i.e. 15-20%, of alfalfa protein inclusion. Feeding with prickly chaff-flower incorporated diets improved the food conversion ratio of rohu (Rao *et al.,* 2006). In contrast, FCR was significantly lower in common carp fed with a diet supplemented with 150 mg/kg of soapbark tree, *Quillaja saponins* (Francis *et al*., 2002). Indeed, Sivarama *et al.* (2004) recorded an improvement in FCR, weight gain and SGF in greasy grouper juveniles fed with diets incorporating 100 and 200 mg/kg diet of Tulsi, *Ocimum sanctum* and Ashwagandha, *W. somnifera*, whereas there was not any improvement in the groups fed with nutmeg, *Myristica fragrans*. Of interest, Glencross *et al.* (2004) declared that incorporation of yellow lupin at 12.5% into fish diets of rainbow trout enhanced FCR.

Feeding with lupin, mango and stinging nettle led to higher feed intake compared to the controls, and this may be attributed to the active ingredients of the plants. Certainly, other studies have recorded a significant decrease in feed intake values of Nile tilapia fed diets containing 33% and 66% plant protein compared to fish fed with 100% plant protein (Fontainhas-fernandes *et al.,* 1999). In previous work with rainbow trout, there was not any effect following an increase in the level of lupin in the diets (Burel *et al.,*

1998; Glencross *et al*., 2002; 2004). In Nile tilapia, replacement of fish meal with plant protein mixture (cottonseed, sunflower, linseed and sesame) by up to 45% had no effect on body weight, body length, weight gain and SGR relative to the fish on a normal (= control) diet (Soltan *et al.,* 2008). The present study demonstrated a slight increase in body composition in treatment groups compared with the controls, without any significant difference between each group and the control. Moreover, the higher crude protein level which was recorded in lupin could be attributed to the high protein content, which reaches 32-36% of the whole seed (Petterson & Mackintosh, 1994). Similar results were observed in rainbow trout fed with 12.5% yellow lupin meal (Glencross *et al.,* 2004). Also, Ali *et al.* (2003) noticed an increase in body composition of Nile tilapia fed with diets containing 5% and 10% alfafa meal, whereas 15% and 20% led to a non significant decrease. In contrast, Bilgin *et al.* (2007) recorded decrease in body moisture, crude protein, crude fat and ash in rainbow trout fed diets containing 20% and 30% hazelnut meal. Moreover, catfish, *Clarias gariepinus*, fed with soybean meal (30% and 60%) and cottonseed meal (30% and 60%) showed decreases in moisture, crude protein or ash content, however the differences were not significant (Toko *et al.*, 2008).

4.5 Effect of long term feeding on digestive enzymes

The growth rate of fish is dependent on the digestive capacity, oxygen availability and/or the metabolic capacity required to support protein synthesis (Blier *et al*., 1997), diet composition and the duration of feeding (Subhadra *et al.,* 2006). Breakdown of large nutrients into small absorbable subunits in the digestive tract of animal depends largely on the available enzymes (Cho, 1987). Digestion of nutrients begins with actions of the (digestive) enzymes in the stomach and continues in the intestine with enzymes secreted by the pancreas, including trypsin, chymotrypsin, amylase and lipase (Nagase 1964; Cockson & Bourne, 1972; Moriarty, 1973; Fang & Chiou, 1989). Moreover, the distribution and intensity of intestinal enzymes varies with feeding habits and intestinal morphology (Kuz'mina & Smirnova, 1992; Sabapathy & Teo, 1993). Studying digestive secretions in fish is difficult because many factors cause variability in the final data:

(1) there is no uniformity in the tissue used for determine the enzymatic activity; consequently, the procedure includes the homogenization of part or the whole digestive tract.

(2) The nutritional status of the animals used in the experiments is not consistent, as the animals are killed either after starvation or at different post-feeding times.
(3) In some cases, the digestive tract is washed before homogenization, whereas others have used the tract and its contents for extraction (Hidalgo *et al.,* 1999).
Moreover, a wide range of techniques has been used to determine the different enzymatic activities (including different substrates, temperature of incubation, and pH), making it difficult to obtain absolute values for the enzymatic activities even from the same species (Hidalgo *et al.,* 1999; Haard & Simpson, 2000). Hofer (1979**)** stated that a change in digestive enzyme concentration can occur within one week.

Generally, a protease is enzyme that conducts proteolysis, which begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. However, there are not any differences in proteolytic activity in omnivorous or carnivorous fish (Hidalgo *et al.,* 1999). Also, Kuz'mina (1990) found a high proteolytic potential in non-carnivorous fish. Vegetable proteins are difficult to digest and although omnivores require comparatively less protein than carnivores, available protein is not so well utilized (Hidalgo *et al.,* 1999). Protease activity has been reported to be correlated with enhancement in food conversion and growth rate in Atlantic cod (Lemieux *et al.,* 1999). Moreover, the pH value of the feed content in the stomach was about 1.5-3 and in the pyloric caeca and intestinal content about 7-9; thus the proteolytic reaction along the digestive tract occurred at suitable pH conditions (Rungruangsak & Utne, 1981).

Generally, the present study recorded increases in total proteases in the stomach in the treatment group at pH 1.5 and 3. Interestingly the highest proteases were recorded at pH 3 where the 2% dose showed significantly higher proteases than the 1% dose. Of relevance, Twinning *et al*. (1982) reported optimum proteases at pH 3 in rainbow trout. In the intestine, the highest total proteases were recorded in the treatment group at pH 9 with no difference at pH 7. Similarly to this study, replacing fish protein with a mixture plant protein (soya bean, alfalfa meal and corn gluten) revealed significantly increased alkaline proteolytic activity of the digestive tract of Nile tilapia, especially at the 50% replacement level (González-Félix *et al.,* 2009). Conversely, redclaw crayfish, *Cherax quadricarinatus,* led to a decrease in total protease in the mid gut in the group fed with lupin meal compared to the groups fed with fish meal (Pavasovic *et al.*, 2007). It is not

surprising that the addition of extra components to fish diets can alter the digestive enzyme activity. Debnath *et al.* (2007) found that increases in total proteases in the digestive tract of rohu correlated with enhanced levels of different crude protein levels in the fish diet. Similarly, Kawai & Ikeda (1973) found increases in total protease activity in the digestive organs of rainbow trout fed with a diet containing a high protein content.

Digestion starts in the stomach by the action of pepsin, and is helped by the acid environment. When the chyme arrives at the intestine, several proteases secreted by the pancreas continue the hydrolysis (Alarácon *et al*., 2001). The results of this study recorded an increase in pepsin activity in the stomach, more so than in the intestine. Certainly, several forms of pepsin reacting at different pH values have been reported (Tanji *et al.*, 1988; Chiu & Pan, 2002). For example, Glass *et al.* (1989) recorded a strong pepsin-like activity at pH 3 in adult halibut and turbot. Also, Sabapathy & Teo (1993) reported higher pepsin activities in the carnivorous sea bass (*Lates calcarifer*) as compared to the herbivorous rabbit fish (*Siganus canaliculatus*).

Amylase is stimulated by glycolytic chains, glycogen, and starch in larval and juvenile fish (Krogdahl *et al*., 2005). Several studies have reported amylase activity in carnivorous fish (Hidalgo *et al*., 1999**;** Alarácon *et al*., 2001; Natalia *et al*., 2004; Furné *et al*., 2005; Suzer *et al.*, 2008). Sabapathy & Teo (1993) and Hidalgo *et al*. **(**1999) documented low amylase activity in carnivorous fish (with a stomach) and high activity in omnivorous fish (without a stomach). It has been suggested that amylase activity depends on the natural diet of each species, with herbivorous and omnivorous fish having more activity than carnivores (Hidalgo *et al*., 1999). The present study recorded an enhancement in amylase activity in all treatment groups. However, the differences were not significant. Similar results were reported by Pavasovic *et al*. (2007), who recorded higher increases in amylase activity in animals fed diets containing plant-based ingredients. For example, amylase activity in animals fed with lupin meal was 4.87 U/mg protein whereas use of fish meal led to 3.63 U/mg protein. Interestingly, Hoyle (1973) and Lopez-Lopez *et al*. (2005) did not find any correlation between amylase activity and carbohydrate content in the diet of lobsters, *Homarus americanus* and red claw crayfish, respectively. Uys & Hecht (1987) attributed the amylase activity in the stomach to exogenous contamination from intestinal activities. Certainly, amylase

activity was found to increase in rainbow trout fed diets containing increased amounts of dietary plant protein (Kawai & Ikeda, 1973).

Lipase is secreted mainly by the pancreas, and exerts a major role in breaking down fats, especially the triacylglycerols, leading to digestion. Generally, the treatment groups recorded higher lipase activity compared to the controls, although there was not any significant difference in the results. However, it was not surprising that the intestine recorded higher lipase activity compared with the stomach because the site of action for lipase is the intestine. These results are in agreement with Pavasovic *et al*. (2007), who recorded higher lipase activity in redclaw fed with soybean meal and lupin meal. They observed a significantly higher digestive enzyme activity in animals fed diets high in plant based ingredients relative to animal-based ones. Moreover, Lopez-Lopez *et al.* (2005) observed significant differences in lipase activity in animals fed a sorghum diet compared to those receiving red crab meal and sardine meal. In contrast, tilapia revealed limited distribution and level of lipase activity in the intestinal tract, possibly due to the low intake of lipid from plant-related diets (Tengjaroenkul *et al*., 2000).

Using lupin, mango and stinging as immunostimulants on fish showed significant enhancement of the innate and adaptive immunity. It will be worthwhile to extract the active components and apply them to rainbow trout to monitor which promotes enhanced resistance to infection and stimulates greatest immunity in the fish. Future work will examine the effect of these active components on cytokine expression, which enhanced the activity of macrophages and monocytes, including interleukin-1 (IL-1), interleukin-8 (IL-8) and tumor necroses factor (TNF) in kidney, liver and gills.

4.6 Conclusions

1- Feeding rainbow trout for 14 days with dietary supplement, i.e. 0.5, 1 and 2 g/ 100 kg food diet of lupin, mango and stinging nettle, led to great resistance against infection with *A. hydrophila.*
2- The plant products led enhancement in cellular and humoral immune responses, specifically phagocytic, respiratory burst, bacteriocidal, complement, lysozyme, antiproteases and MPO activities, and total protein levels.
3- The higher doses (1% and 2%) led to great effects in term of enhancement of the immune system and resistance to *A. hydrophila*.

4- The plant products, especially stinging nettle, stimulated immune cells of the head kidney to induce the expression of cytokines gene IL-1ß, IL-8 and TGF-ß.
5- Haematological studies recorded increases in WBC, RBC, Hct and Hb following the administration of the plant dietary supplements.
6- The plant products stimulated growth rate in rainbow trout.
7- The dietary supplements led to increases in digestive enzymes including total proteases, pepsin, amylase and lipase.

References

Abbass, A., Sharifuzzaman, S.M. and Austin, B. 2010. Cellular components of probiotics control *Yersinia ruckeri* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. **33**, 31-37.

Abdel-Aal, W.E., Abdel-Nabi, I.M., Hanna, R.A. and Mahdy, K.A. 1993. Biochemical and histological changes in normal and alloxan-diabetic rats after lupin seeds administration. J. Egypt. Ger. Soc. Zool. **10**, 251-267.

Abdelkhalek, N.K., Komiya, A., Kato-Unoki, Y., Somamoto, T. and Nakao. M. 2009. Molecular evidence for the existence of two distinct IL-8 lineages of teleost CXC-chemokines. Fish Shellfish Immunol. **27,** 763-767

Adams, A., Auchinachie, N., Bundy, A., Tatner, M.F. and Horne, M.T. 1988. The potency of adjuvanted injected vaccines in rainbow trout (*Salmo gairdneri* Richardson) and bath vaccines in Atlantic salmon (*Salmo salar* L.) against furunculosis. Aquaculture. **69**, 15-26.

Afanasjev, V.I. and Afanasjev, G.V. 2001. Using immune modulators in industrial fish-breeding. Fish Breed. Fishery. **3**, 34-36 (abstract).

Afonso, A., Ellis, A.E. and Silva, M.T. 1997. The leucocyte population of the unstimulated peritoneal cavity of rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. **7**, 335-348.

Afuang, W., Siddhuraju, P. and Becker, K. 2003. Comparative nutritional evaluation of raw, methanol extracted residues and methanol extracts of moringa (*Moringa oleiera* lam.) leaves on growth performance and feed utilisation in Nile tilapia (*Oreochromis niloticus* L.). Aquacult. Res. **34,** 1147-1159.

Agius, C., Horne, M.T. and Ward, P.D., 1983. Immunisation of rainbow trout, *Salmo gairdneri* Richardson, against vibriosis: Comparison of an extract antigen with whole cell bacterians by oral and intraperitoneal routes. J. Fish. Dis. **6**, 129-134.

Ai, Q., Mai, K., Tan, B., Xu, W., Zhang, W., Ma, H. and Liufu, Z. 2007. Effects of dietary vitamin C on survival, growth, and immunity of large yellow croaker, *Pseudosciaena crocea*. Aquaculture **261**, 327-336.

Akbay, P., Basaran, A.A., Undeger, U. and Basaran, N. 2003. In vitro immunomodulatory activity of flavonoid glycosides from *Urtica dioica*. Phytother. Res. **17**, 34-37.

Alarácon, F.J., Garcıa-Carreño, F.L. and Navarrete del Toro, M.A. 2001. Effect of plant protease inhibitors on digestive proteases in two fish species, *Lutjanus argentiventris* and *L. novemfasciatus*. Fish Physiol. Biochem. **24**, 179-189.

Albrektsen, S., Mundheim, H. and Aksnes, A. 2006. Growth, feed efficiency, digestibility and nutrient distribution in Atlantic cod (*Gadus morhua*) fed two different fish meal qualities at three dietary levels of vegetable protein sources. Aquaculture **261**, 626-640.

Alexander J.B., and Ingram, G.A. 1992. Non cellular nonspecific defense mechanisms of fish. Annu. Rev. Fish Dis. **2**, 249-279.

Ali, A., Al-Asgahn, N.A., Al-Ogaily, M.S. and Ali, S. 2003. Effect of Feeding Different Levels of Alfalfa Meal on the Growth Performance and Body Composition of Nile Tilapia (*Oreochromis niloticus*) Fingerlings. Asian Fish. Sci. **16**, 59-67.

Allen, D.A., Austin, B. and Colwell, R.R. 1983. Numerical taxonomy of bacterial isolates associated with a fresh water fisher. J. Gen. Microbiol. **129**, 2043-2062.

Amin, A.B., Mortensen, L. and Poppe, T.T. 1992. Histology Atlas of Normal Structure of Salmonids. Akvapatologisk Laboratorium, Norway.

Anbarasu, K., Thangakrishnan, K., Arun, B.V. and Chandran, M.R. 1998. Assessment of immune response in freshwater catfish (*Mystus vittatus* Bloch) to different bacterins of *Aeromonas hydrophila*. Ind. J. Exp. Biol. **36**, 990-995.

Anderson, D.P. 1992. Immunostimulants, adjuvants and vaccine carriers in fish: applications to aquaculture. Annu. Rev. Fish. Dis. **2**, 281-307.

Anderson, D.P. and Siwicki, A.K. 1994. Duration of protection against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion. Prog. Fish Cult. **56**, 258-261.

Anderson, D.P., Siwicki, A.K. and Rumsey, G.L. 1995. Injection or immersion delivery of selected immunostimulants to trout demonstrate enhancement of nonspecific defense mechanisms and protective immunity. In: Shariff, M., Subasighe, R.P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture Vol. 11. Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 413-426.

Anderson, M., Amemiya, C., Luer, C., Litman, R., Rast, J., Niimura, Y. and Litman, G. 1994. Complete genomic sequence and patterns of transcription of a member of an unusual family of closely related, chromosomally dispersed Ig gene clusters in Raja. Int. Immunol. **6**, 1661-1670.

Anila, L. and Vijayalakshmi, N.R. 2003. Antioxidant action of flavenoids from *Mangifera indica* and *Emblica officinalis* in hypercholesterolemic rats. Fd. Chem. **83**, 569-574.

Ansary, A., Haneef, R.M., Torres, J.L. and Yadav, M. 1992. Plasmids and antibiotic resistance in *A. hydrophila*. J. Fish Biol. **15**, 191-196.

AOAC (Association of Official Analytical Chemists). 1990. Official Methods of Analysis of the Association of Official Analytical Chemists, 15th ed. Association of Official Analytical Chemists, Washington, DC, USA.

Aoki, T. 1988. Drug-resistant plasmids from fish pathogens. Microbiol. Sci. **5,** 219-223.

Aoki, T. and Egusa, S. 1971. Detection of Resistance Factors in Fish Pathogen *Aeromonas liquefaciens*. J. Gen. Microbiol. **65**, 343-349.

Aranishi, F. and Nakane, M. 1997. Epidermal proteases of the Japanese eel. Fish Physiol. Biochem. **16**, 471-478.

Aranishi, F. 1999. Purification and characterization of alpha (1)-proteinase inhibitor from carp (*Cyprinus carpio*) serum. Mar. Biotechnol. **1**, 33-43.

Arason, G.J. 1996. Lectins as defence molecules in vertebrates and invertebrates. Fish Shellfish Immunol. **6**, 277-289.

Ardó, L., Yin, G., Xu, P., Váradi, L., Szigeti, G., Jeney, Z. and Jeney, G. 2008. Chinese herbs (*Astragalus membranaceus* and *Lonicera japonica*) and boron enhance the non-specific immune response of Nile tilapia (*Oreochromis niloticus*) and resistance against *Aeromonas hydrophila*. Aquaculture **275**, 26-33.

Aruna, B.V. and Chandran, M.R. 1996. Efficacy of CIFAX and herbal mixture in the prophylaxis of EUS. National Seminar on Diseases in Aquaculture, Kakinada, India.

Ates, B., Orun, I., Talas, Z.S., Durmaz, G. and Yilmaz I. 2008. Effects of sodium selenite on some biochemical and hematological parameters of rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) exposed to Pb^{2+} and Cu^{2+}. Fish Physiol. Biochem. **34**, 53-59.

Austin, B. and Austin, D.A. 2007. Bacterial Fish Pathogens, Disease of Farmed and Wild Fish, 4th ed. Springer Praxis, Godalming.

Austin, B. 2005. Bacterial pathogens of marine fish. In: Oceans and Health, Pathogens in the Marine Environment (Ed. Belkin and Colwell) Springer. New York.

Austin, B. and Mcintosh, D. 1988. Natural antimicrobial compounds on the surface of rainbow trout. J. Fish Dis. **15**, 275-277.

Austin, B. and Day, J.G. 1990. Inhibition of prawn pathogenic *Vibrio* spp by a commercial spray-dried preparation of *Tetraselmis suecica*. Aquaculture. **90**, 389-392.

Austin, B. and Austin, D.A., 1989. Microbiological Examination of Fish and Shellfish. Ellis Horwood, Chichester.

Austin, B., Stuckey, L.F., Robertson, P.A.W., Effendi, I. and Griffith, D.R.W. 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. J. Fish Dis. **1,** 93-96.

Awaad, S.A., Maitland, D.J. and Moneir, S. 2007. New alkaloids from *Casimiroa edulis* fruits and their pharmacological activity. Chem. Nat. Comp. **43**, 576-580.

Awaad, A.S. 2009. Flavonoids of Bidens bipinnata and their Antioxidant Activity. J. King Saud Univ. **21**, 183-189.

Awaad, S.A. and Al-Jaber, N.A. 2010. Antioxidant of natural plants. Ethnomed. Source Mech. **27**, 1-35.

Azizoglu, A. and Cengizler, I. 1996. An investigation on determination of some hematologic parameters in healthy *Oreochromis niloticus* (L.). Turk. J. Vet. Ani. Sci. **20**, 425-431.

Baba, T., Watase, Y. and Yoshinaga, Y. 1993. Activation of mononuclear phagocyte function by levamisole immersion in carp. Nipp. Suis. Gakk. **59**, 301-307.

Bagni, M., Romano, N., Finoia, M.G., Abelli, L., Scapigliati, G., Tiscar, P.G., Sarti, M. and Marino, G. 2005. Short and long-term effects of a dietary yeast ß-glucan (Macrogard) and alginic acid (Ergosan) preparation on immune response in sea bass (*Dicentrarchus labrax*). Fish Shellfish Immunol. **18**, 311-325.

Balcázar**,** J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D. and Muzquiz, J.L. 2006. The role of probiotics in aquaculture. Vet. Microbiol. **114**, 173-186.

Bangoula, D., Parent, J.P. and Vellas, F. 1993. Nutritive value of white lupin (*Lupinus albus* var Lutop) in rainbow trout (*Oncorhynchus mykiss*). Effects of extrusion cooking. Reprod. Nutr. Dev. **33**, 325-334.

Baticados, M.C.L., Lavilla-Pitogo, E.R., Cruz-Lacierda de la Pena, L.D. and Sunaz, N.A. 1990. Studies the chemical control of luminous bacteria *Vibrio harveyi* and *V. splendidus* isolated from diseased Penaeous monodon larvae and rearing water. Dis. Aquat. Org. **9**, 133-139.

Bhadauria, M., Jadon, A. Sharma, A. and Shukla, S. 2002. Effect of propriety herbal formulation against chronic carbon tetrachloride induced hepatotoxicity. Ind. J. Exp. Biol. **40,** 1254-1259.

Bilgin, Ö., Türker, A. and Tekinay, A.A. 2007. The use of hazelnut meal as a substitute for soybean meal in the diets of rainbow trout (*Oncorhynchus mykiss*) Turk. J. Vet. Anim. Sci. **31**, 145-151.

Bird, S., Wang, T., Zou, J., Cunningham, C. and Secombes, C.J. 2002. The first cytokine sequence within cartilaginous fish: IL-1ß in the small spotted catshark (*Scyliorhinus canicula*). J. Immunol. **168**, 3329- 3340.

Blaxhall, P.C. 1972. The hematological assessment of the health of freshwater fish. A review of selected literature. J. Fish. Biol. **4,** 593-604.

Blazer, V.S. 1992. Nutrition and disease resistance in fish. Annu. Rev. Fish Dis. **1**, 309-323.

Blazer, V.S. and Wolke, R.E. 1984. The effects of atocopherol on immune response and non-specific resistance factors of' rainbow trout (*Salmo gairdneri* Richardson). Aquaculture **37**, 1-9.

Blier, P., Pelletier, D. and Dutil, J.D. 1997. Does aerobic capacity set a limit on fish growth rate? Rev. Fish. Sci. **5**, 323-340.

Bowden, T.J., Butler, R., Bricknell, I.R. and Ellis, A.E. 1997. Serum trypsin-inhibitory activity in five species of farmed fish. Fish Shellfish Immunol. **7**, 377-385.

Bradford, M.M. 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal. Biochemist. **72**, 248-254.

Brown, B.A. 1988. Routine hematology procedures. In Hematology: Principles and Procedures. Leo and Febiger, Philadelphia. pp. 7-122.

Brunt, J. and Austin, B. 2005. Use of a probiotics to control lactococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. **28**, 693-701.

Burel, C., Boujard, T., Corraze, G., Kaushik, S.J., Boeuf, G., Mol, K.A., Van der Geyten, S. and Kuhn, E.R. 1998. Incorporation of high levels of extruded lupin in diets for rainbow trout (*Oncorhynchus mykiss*): nutritional value and effect on thyroid status. Aquaculture **163**, 325-345.

Burel, C., Boujard, T., Tulli, F. and Kaushik, S. 2000. Digestibility of extruded peas, extruded lupin, and rapeseed meal in rainbow trout (*Oncorhynchus mykiss*) and turbot (*Psetta maxima*). Aquaculture **188**, 285-298.

Chandran, M.R., Aruna, B.V. Logambal, S.M. and Dinakaran M.R. 2002. Immunisation of Indian major carps against *Aeromonas hydrophila* by intraperitoneal injection. Fish Shellfish Immunol. **13**, 1-9.

Chang, B.J. and Bolton, S.M. 1987. Plasmids and resistance to antimicrobial agents in *Aeromonas sobria* and *Aeromonas hydrophila* clinical isolates. Antimicrob. Ag. Chemother. **31**, 1281- 1282.

Chansue, N., Endo, M. Kono, T. and Sakai, M. 2000. The stimulation of cytokine-like proteins in tilapia (*Oreochromis niloticus*) orally treated with ß-1, 3-glucan. Asian Fish. Sci. **13**, 271-278.

Chaurasia, N. and Wichtl, M. 1987. Flavonol glycosides from *Urtica dioica*. Planta Med. **53**, 432-434.

Chaurasia, N. and Wichtl, M. 1986a. Phenylpropane und Lignane aus der Wurzel von *Urtica dioica* L. Dtsch. Apothek. Ztg. **126**, 1559-1563.

Chaurasia, N. and Wichtl, M. 1986b. Scopoletin, 3-[beta]-Sitosterin und Sitosterin-3-[beta]-D-glucosid aus Brennesselwurzel (*Urticae radix*). Dtsch. Apothek. Ztg. **126**, 81-83.

Chen, D., and Ainsworth, A.J. 1992. Glucan administration potentiates immune defense mechanisms of channel catfish, *Ictalurus punctatus* Rafinesque. J. Fish Dis. **15**, 295-304.

Chen, L., He, C., Baoprasertkul, P., Xu, P., Li, P., Serapion, J., Waldbieser, G., Wolters, W. and Liu, Z. 2005. Analysis of a catfish gene resembling interleukin-8: cDNA cloning, gene structure, and expression after infection with *Edwardsiella ictaluri*. Devel. Comp. Immunol. **29**, 135-142.

Cheng, A., Chen, Y. and Chen, J. 2008. Dietary administration of sodium alginate and k-carrageenan enhances the innate immune response of brown-marbled grouper *Epinephelus fuscoguttatus* and its resistance against *Vibrio alginolyticus.* Vet. Immunol. Immunol. **121**, 206-215.

Chiu, S.T. and Pan, B.S. 2002. Digestive protease activities of juvenile and adult eel (*Anguilla japonica*). Aquaculture **205**, 141-156.

Cho, C.Y. 1987. The enery in fish nutrition In: Nutrtion in Aquaculture, Espinosa de los Monteros, J., Labarta, U. (Eds.), vol. II. CAICYT, Madrid, pp. 197-244.

Christybapita, D., Divyagnaneswari, M. and Michael, D. 2007. Oral administration of *Eclipta alba* leaf aqueous extract enhances the non-specific immune responses and disease resistance of *Oreochromis mossambicus*. Fish Shellfish Immunol. **23,** 840-852.

Chrubasik, J.E., Roufogalis, B.D., Wagner, H. and Chrubasik, S. 2007. A comprehensive review on the stinging nettle effect and efficacy profiles. Part II: *Urticae radix*. Phytomedicine**. 14**, 568-579.

Chung, S. and Secombes, C.J. 1988. Analysis of events occurring within teleost macrophages during the respiratory burst. Comp. Bioch. Physiol. **89**, 539-544.

Cipriano, R.C. Bullock, G.L. and Pyle, S.W. 2001. *Aeromonas hydrophila* and motile *Aeromonad septicemias* of fish. Fish Disease leaflet 68, US Department of the Interior Fish & Wildlife Service. Washington.

Citarasu, T., Sivaram, V., Immanuel, G., Rout, N. and Murugan, V. 2006. Influence of selected Indian immunostimulant herbs against white spot syndrome virus (WSSV)

infection in black tiger shrimp, *Penaeus monodon* with reference to haematological, biochemical and immunological changes. Fish Shellfish Immunol. **21**, 372-384.

Claire, M., Holland, H. and John, D.L. 2002. The complement system in teleosts. Fish Shellfish Immunol. **12**, 399-420.

Clark, N.M. and Chenoweth, C.E. 2003. *Aeromonas* infection of the hepatobiliary system: report of 15 cases and review of the literature. Clin. Infect. Dis. **37**, 506-513.

Clem, L.W. 1971. Phylogeny of immunoglobulin structure and function. IV. Immunoglobulins of the giant grouper, *Epinephalus itaira*. J. Biol. Chem. **246**, 9-15.

Cockson, A. and Bourne, D. 1972. Enzymes in the digestive tract of two species of euryhaline fish. Comp. Biochem. Physiol. **41**, 715-718.

Cojocaru, M., Droby, S., Glotter, E., Goldman, A., Gottlieb, H.E. and Jacoby, B. 1986. 5-(12-heptadecenyl)-resorcinol, the major component of the antifugal activity in the peel of mango fruit. Phytochemistry **25,** 1093–1095.

Cole, A.M., Weis, P. and Dizmond, G. 1997. Isolation and characterisation of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. J. Biol. Chem. **272,** 12008–12013.

Coppo, J.A. 2001. Internal Environment comparative physiology. Ed Dunken, Buenos Aires (Argentina). pp. 212-216.

Corripio-Miyar, Y., Bird, S., Tsamopoulos, K. and Secombes, C.J. 2006. Cloning and expression analysis of two pro-inflammatory cytokines, IL-1beta and IL-8, in haddock (*Melanogrammus aeglefinus*). Molecul. Immuno. **44,** 1361-73.

Cuesta, A., Ortuno, J., Rodriguez, A., Esteban, M.A. and Meseguer, J. 2002. Changes in some innate defence parameters of seabream (*Sparus aurata* L.) induced by retinol acetate. Fish Shellfish Immunol. **13**, 279-291.

Dalev, P., Ljubomirova, A. and Ivanov, I. 1996. Enzyme extraction, functional properties and application of leaf protein fractions of nettle. Biotechnol. Lett. **18**, 107-110.

Dalmo, R.A., Ingebrightsen, K. and Bogwald, J. 1997. Non-specific defense mechanisms in fish, with particular reference to the reticuloendothelial system (RES). J. Fish Dis. **20**, 241-73.

Danilova, N., Bussmann, J., Jekosch, K. and Steiner, L.A., 2005. The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. Nat. Immunol. **6,** 295–302

Davis, H.S. 1956. Culture and diseases of game fishes. University of California Press, Berkeley.

Davis, J.F. and Hayasaka, S.S., 1984. The enhancement of resistance of the American eel, *Anguilla rostrata* Le Sueur, to a pathogenic bacterium *Aeromonas hydrophila*, by an extract of the tunicate *Ecteinascidia turbinata*. J. Fish Dis. **7**, 311-316.

Davis, W.A., Kane, J.G. and Garagusi, V.F. 1978. Human *Aeromonas* infections: a review of the literature and a case report of endocarditis. J. Med. **57**, 267 - 277.

De Baulny, M.O., Quentel, C., Fournier, V., Lamour, F. and Le Gouvello, R. 1996. Effect of long-term oral administration of ß-glucan as an immunostimulant or an adjuvant on some non-specific parameters of' the immune response of turbot *Scophthalmus maximus*. Dis. Aquat. Org. **26**, 139-147.

De Figueredo, J. and Plumb, J.A. 1977. Virulence of different isolation of *Aeromonas hydrophila* in channel catfish. Aquaculture **11**, 349- 354.

De Haas, C.J.C., van Leeuwen, E.M.M., van Bommel, T., Verhoef, J., van Kessel, K.P. and van Strijp, J.A. 2000. Serum amyloid P component bound to Gram-negative bacteria prevents lipopolysaccharide-mediated classical pathway complement activation. Infect. Immun. **68**,1753-1759.

De Paola, A.T. 1988. Phenotypic and genotypic characterization of tetracycline- and oxytracycline- resistant *Aeromonas hydrophila* from cultured channel catfish (*Ictalurus punctatus*) and their environment. Appl. Environ. Microbiol. **54**, 1861- 1863.

De Schrijver, R. and Olievier, F. 2000. Protein digestion in juvenile turbot (*Scophthalmus maximus*) and effects of dietary administration of *Vibrio proteolyticus*. Aquaculture. **186**, 107–116.

Debnath, D., Pal, A.K., Sahu, N.P., Yengkokpam, S., Baruah, K., Choudhury, D. and Venkateshwarlu, G. 2007. Digestive enzymes and metabolic profile of *Labeo rohita* fingerlings fed diets with different crude protein levels. Comp. Bioch. Physiol. **146**, 107-114.

Díaz-Rosales, P., Salinas, I., Rodríguez, A., Cuesta, A., Chabrillon, M., Carmen, M., Balebona, M., Moriñigo, A., Esteban, A. and Meseguer, J. 2006. Gilthead seabream (*Sparus aurata* L.) innate immune response after dietary administration of heat-inactivated potential probiotics. Fish Shellfish Immunol. **20**, 482-492.

Divyagnaneswari, M., Christybapita, D. and Michael, R.D. 2007. Enhancement of nonspecific immunity and disease resistance in *Oreochromis mossambicus* by *Solanum trilobatum* leaf fractions. Fish Shellfish Immunol. **23**, 249-259.

Dopazo, C.P., Lemos, M.L., Lodeiros, C., Bohnches, J., Barja, J.L. and Toranzo, A.E. (1988). Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. J. Appl. Bacteriol. **65,** 97-101.

Duff, D.C.B. 1942. The oral immunisation of trout against *Bacterium salmonicida*. J. Immunol. **44**, 81-94.

Dügenci, S.K., Arda, N. and Candan, A. 2003. Some medicinal plants as immunostimulant for fish. J. Ethnopharmacol. **88**, 99-106.

Duncan, P.L. and Klesius, P.H. 1996. Dietary immunostimulants enhance nonspecific immune responses in channel catfish but not resistance to *Edwardsiella ictaluri*. J. Aquat. Anim. Hlth. **8**, 241-248.

Duttaa, S., Sinhab, B., Bhattacharyab, B., Chatterjeeb, B. and Mazumder, S. 2005. Characterization of a galactose binding serum lectin from the Indian catfish, Clarias batrachus: Possible involvement of fish lectins in differential recognition of pathogens. Comp. Biochem. Physiol. **141**, 76-84.

Edahiro, T., Hamoguchi, M. and Kusuda, R. 1990. Effect of glycyrrhizine against streptococcal infection of young yellowtail, *Seriola quinqueradiata*. Suisanzoshoku **38**, 239–243 (In Japanese).

Egwurugwu, J.N., Ufearo, C.S., Abanobi, O.C., Nwokocha, C.R., Duruibe, J.O., Adeleye, G.S., Ebunlomo, A.O., Odetola, A.O. and Onwufuji, O. 2007. Effects of ginger (*Zingiber officinale*) on cadmium toxicity. African J. Biotech. **6,** 2078-2082.

El- Ashram, A.M.M. and El-Boshy, M.E. 2008. Assessement of dietary bovine lactoferrin in enhancement of immune function ad disease resistance in Nile tilapia (*Oreochromis nilcoticus*). 8th International Symposium on Tilapia in Aquaculture. 1097-1107.

Ellis, A.E. 1987. Inhibition of the *Aeromonas salmonicida* extracellular protease by α-2 macroglobulin in the serum of rainbow trout. Microb. Pathog. **3**, 167–177.

Ellis A.E. 2001. Innate host defence mechanism of fish against viruses and bacteria. Dev. Comp. Immunol. **25**, 827-839.

Ellis, A.E. 1999. Immunity to bacteria in fish. Fish Shellfish Immunol. **9**, 291-308.

El-Nattat, W.S. and El-Kady, R.I. 2007. Effect of different medicinal plant seeds residues on the nutritional and reproductive performance of adult male rabbits. J. Agri. Bio. **3**, 479-485.

El-Sayed, A.F.M., 1999. Alternative dietary protein sources for farmed tilapia, *Oreochromis* spp. Aquaculture **179**, 149-168.

Elward, K., and Gasque, P. 2003. ‘‘Eat me’’ and ‘‘don’t eat me’’ signals govern the innate immune response and tissue repair in the CNS: emphasis on the critical role of the complement system. Mol. Immunol. **40**, 85-94.

Engstad, R.E., Robertson, B. and Frivold, E. 1992. Yeast glucan induces increase in lysozyme and complement-mediated haemolytic activity in Atlantic salmon blood. Fish Shellfish Immunol. **2**, 287-297.

Erdal, J.I., Evensen, O., Kaursted, O.K., Lillehaug, A., Solbakken, R. and Thorud, K., 1991. Relationship between diet and immune response in Atlantic salmon (*Salmo salar* L.) after feeding various levels of ascorbic acid and omega-3 fatty acids. Aquaculture **98**, 363-379.

Eskander, E.F. and Won Jon, H. 1995. Hypoglycemic and hyperinsulinemic effects of some Egyptian herbs used for the treatment of diabetes mellitus (type (2) in rats. Egypt J. Pharm. Sci. **36**, 331-342.

Espe, M., Lemme, A., Petri, A. and El-Mowafi, A., 2007. Assessment of lysine requirement for maximal protein accretion in Atlantic salmon using plant protein diets. Aquaculture **263**, 168-178.

Evelyn, T.P. 1997. A historical review of fish vaccinology. Dev. Biol. Stand. **90**, 3-12.

Faktorovich, K.A. 1969. Histological changes in the liver, kidneys, skin and brain of fish sick with red rot. Pages 83-101 in Infectious diseases of fish and their control. Division of Fisheries Research, Bureau of Sport Fisheries and Wildlife. Washington, D. C. Translated from the Russian by R. M. Howland.

Fang, L.S. and Chiou, S.F. 1989. Effect of salinity on the activities of digestive proteases from the tilapia fish, *Oreochromis niloticus* in different culture environments. Comp. Biochem. Physiol. **93**, 439-443.

Farhangi, M. and Carter, C.G. 2001. Growth, physiological and immunological responses of rainbow trout (*Oncorhynchus mykiss*) to different dietary inclusion levels of dehulled lupin (*Lupinus angustifolius*). Aqua. Res. **32**, 329- 340.

Faruk, M.A.R., Sarker, M.M.R., Alam, M.J. and Kabir, M.B. 2004. Economic loss from fish diseases on rural freshwater aquaculture of Bangladesh. Pakistan J. Bio. Sci. **7**, 2086-2091.

Fast, M.D., Sims, D.E., Burka, J.F., Mustafa, A. and Ross, N.W. 2002. Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. Comp. Biochem. Physiol. **132**,645-57.

Fellah, J.S. Kerfourn, F. Wiles, M.V., Schwager, J. and Charlemagne, J. 1993. Phylogeny of immunoglobulin heavy chain isotypes: Structure of the constant region of *Ambystoma mexicanum* ~ chain deduced from cDNA sequence. Immunogenetics **38**, 311-317.

Fjalestad, K.T., Gjedrem, T. and Gjerde, B. 1993. Genetic improvement of disease resistance in fish: on overview. Aquaculture **111**, 65-74.

Fontainhas-fernandes, A., Gomes, E., Reis-Henriques, M. and Coimbra, J. 1999. Replacement of fish meal by plant proteins in the diet of Nile tilapia: digestibility and growth performance. Aqua. Int. **7,** 57-67.

Fournier, V., Huelvan, C. and Desbruyeres, E. 2004. Incorporation of a mixture of plant feedstuffs as substitute for fish meal in diets of juvenile turbot (*Psetta maxima*). Aquaculture **236**, 451-465.

Francis, G., Makkar, H.P. and Becker, K. 2002. Dietary supplementation with a *Quillaja saponin* mixture improves growth performance and metabolic efficiency in common carp (*Cyprinus carpio* L.). Aquaculture. **203**, 311-320.

Freedman, S.J. 1991. The role of α2 -macroglobulin in furunculosis: a comparison of rainbow trout and brook trout. Comp. Biochem. Physiol. **98**, 549-553.

Fujii, T., Nakamura, T., Sekizawa, A. and Tomonaga, S. (1992). Isolation and characterization of a protein form hagfish serum that is homologous to the third component of the mammalian complement system. J. Immunol. **148**, 117-123.

Fujiki, K., Nakao, M., Shin, D. and Yano, T. 1998. cDNA cloning of a carp homologue of mammalian interleukin-1ß. European Molecular Biology Laboratory accession no. AB010701.

Fuller, R. 1989. Probiotics in man and animals. J. Appl. Bacteriol. **66**, 365-378.

Fuller, R. 1997. Probiotics 2: Applications and practical aspects (Fuller, R. Ed.). London: Chapman and Hall. pp, 1-9.

Furné, M., Hidalgo, M.C., Lo´pez, A., Garcı´a-Gallego, M., Morales, A.E., Domezain, A., Domezaine´, J. and Sanz, A. 2005. Digestive enzyme activities in Adriatic sturgeon *Acipenser naccarii* and rainbow trout *Oncorhynchus mykiss*. A comparative study. Aquaculture **250**, 391– 398.

Galeotti, M. 1998. Some aspects of the application of immunostimulants and a critical review of methods for their evaluation. J. Appl. Ichthyol. **14**, 189-199.

Galeotti. M., Volpatti, D. and Jeney, G. 1995. The nature of non-specific immune response of sea bass (*Dicentrarchus labrax*) to *Pasteurella piscicida* following bath exposure to levamisole. European Assoc. Fish Pathologists. Seventh Int. Conf. Palma de Mallorca, Spain.

Galina, J., Yin, G., Ardo, L. and Jeney, Z. 2009. The use of immunostimulating herbs in fish. An overview of research. Fish Physiol. Biochem. **35**, 669-676.

Galindo-Villegas, J. and Hosokawa, H. 2004. Immunostimulants: towards temporary prevention of diseases in marine fish. In: Cruz Suarez, L. E., Ricque Marie, D., Nieto Lopez, M. G., Villarreal, D., Scholz, U. and Gonzalez, M. 2004. *Avances en Nutricion Acuicola VII. Memorias del VII Simposium Internacional de Nutricion Acuicola*. 16-19. Hermosillo, Sonora, Mexico.

Gan, F.L. 1998. Basic immunology. Hubei Publishing House of Sciences and Technologies. pp. 421–423 (Chinese).

Gannam, A.L. and Schrock, R.M. 1999. Immunostimulants in fish diets. J. Appl. Aqua. **9,** 53- 89.

Garcia, F., Pilarski, F., Makoto E., Moraes, F. and Martins, M.L. 2007. Hematology of *Piaractus mesopotamicus* fed diets supplemented with vitamins C and E, challenged by *Aeromonas hydrophila*. Aquaculture **271**, 39–46.

Garrido, G., Delgado, R., Lemus, Y., Rodriguez, J., Garcia, D. and Nunze-Selles, A.J. 2004b. Protection against septic shock and suppression of tumor necrosis factor alpha and nitric oxide production on macrophage and microgila by the standard aqueous extract of *Mangifera indica* L. Pharmacol. Res. **50,** 165-172.

Garrido, G., Gonzalez, D., Lemus, Y., Garcia, D., Lodeiro, L. and Quintero, G. 2004a. In vivo and in vitro anti-inflammatory activity of *Mangifera indica* L. extract. Pharmacol. Res. **50**, 143-149.

Garriques, D., and Arevalo, G. (1995). An evaluation of the production and use of a live bacterial isolate to manipulate the microbial flora in the commercial production of Penaeus vannzamei postlarvae in Ecuador. In: Swimming through troubled water, proceedings of the special session on shrimp farming, pp 53–59 (C.L. Browdy, and J.S. Hopkins, Eds.).World Aquaculture Society, Louisiana State University, USA.

Gatlin, D.M. and Wilson, R.P. 1983. Dietary zinc requirement of fingerling channel catfish. J. Nutr. **113**, 630-635.

Gerwich, L., Steinhauer, R., Lapatra, S., Sandell, T., Ortuno, J. and Hajiseyedjavadi, N. 2002. The acute phase response of rainbow trout (*Oncorhynchus mykiss*) plasma protein to viral, bacterial and fungal inflammatory agents. Fish Shellfish Immunol. **12**, 299-242.

Ghanem, E.H., Mussa, M.E. and Eraki, H.M. 1993. *Aeromonas*-associated gastroenteritis in Egypt. Zbl. Mikrobiol. **148**, 441-447.

Gibbs, E.L. 1963. An effective treatment for red-leg disease in *Rana pipiens*. Lab. Animal Care **13**, 781-783.

Gildberg, A., Johanson, A. and Jogwald, J. 1995. Growth and survival of Atlantic salmon (*Salmo salar*) fry given diets supplemented with fish protein hydrolysate and

lactic acid bacteria during a challenge trial with *Aeromonas salmonicida*. Aquaculture **138**, 23-34.

Gioacchini, G., Smith, P. and Carnevali, O. 2008. Effects of Ergosan on the expression of cytokine genes in the liver of juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to enteric redmouth vaccine. Vet. Immunol. Immunopathol. **123**, 215-222.

Glass, H.D., MacDonald, N.L., Moran, R.M. and Stark, J.R. 1989. Digestion of protein in different marine species. Comp. Biochem. Physiol. **94**, 607- 611.

Glencross, B., Curnow, J.G., Hawkins, W.E. and Felsing, M. 2002. Preliminary evaluation of yellow lupin (*Lupinus luteus*) kernel meal as an alternative protein resource in the diet of sea-cage reared rainbow trout, *Oncorhynchus mykiss*. J. Wld. Aqua. Soc. **33**, 287- 296.

Glencross, B., Evans, D., Hawkins, W. and Jones, B. 2004. Evaluation of dietary inclusion of yellow lupin (*Lupinus luteus*) kernel meal on the growth, feed utilisation and tissue histology of rainbow trout (*Oncorhynchus mykiss*). Aquaculture **235**, 411-422.

Glencross, B., Hawkins, W., Evans, D., Rutherford, N., Dods, K., McCafferty, P. and Sipsas, S. 2007. Evaluation of the influence of drying process on the nutritional value of lupin protein concentrates when fed to rainbow trout (*Oncorhynchus mykiss*). Aquaculture **265**, 218-229.

Gomes, E.F., Rema, P. and Kaushik, S.J. 1995. Replacement of fishmeal by plant proteins in the diet of rainbow trout (*Oncorhynchus mykiss*): digestibility and growth performance. Aquaculture **130**, 177-186.

González-Félix, M.L., Castillo-Yanez, F.J., Ocańo-Higuera, V.M., Perez-Velazquez, M., Cota-Moreno, V. and Lozano-Taylor, J. 2009. Effect of dietary protein source and time on alkaline proteolytic activity of Nile tilapia (*Oreochromis niloticus*). Fish Physiol. Biochem. **10,** 1-7.

Gopalakannan, A. and Arul, V. 2006. Immunomodulatory effects of dietary intake of chitin, chitosan and levamisole on the immune system of *Cyprinus carpio* and control of *Aeromonas hydrophila* infection in ponds. Aquaculture **255**, 179-187.

Gouveia, A., Oliva-Teles, A., Gomes, E. and Rema, P. 1993. Effect of cooking/expansion of three legume seeds on growth and food utilisation by rainbow trout. In: Kaushik, S.J., Luquet, P. (Eds.), Fish Nutrition in Practice. INRA, Paris, 933-938.

Grinde, B., Lie, O., Poppe, T. and Salte, R. (1988). Species and individual variation in lysozyme activity in fish of interest in aquaculture. Aquaculture **68**, 299- 304.

Grinde, B. 1989. Lysozyme from rainbow trout, *Salmo gairdneri* Richardson, as an antibacterial agent against fish pathogens. J. Fish Dis. **12**, 95-104.

Groberg, W.J., McCoy, R.H., Pilcher, K.S. and Fryer, J.L. 1978. Relation of water temperature to infections of coho salmon (*Oncorhynchus kisutch*), chinook salmon (*O. tshawytscha*), and steelhead trout (*Salmo garidneri*) with *Aeromonas salmonicida* and *A. hydrophila*. J. Fish. Res. Bd. Can. **35**, 1-7.

Gülçin, I., Küfrevioglu, I., Oktay, M. and Büyükokuroglu, E.M. 2004. Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (*Urtica dioica* L.) J. Ethnopharmacol. **90**, 205-215.

Haard, N.F. and Simpson, B.K. 2000. Seafood enzymes: utilization and influence on postharvest seafood quality. Marcel Dekker, New York, pp. 670.

Haddad, G., Hanington, P.C., Wilson, E.C., Grayfer, L. and Belosevic, M. 2008. Molecular and functional characterization of goldfish (*Carassius auratus* L.) transforming growth factor beta. Devel. Comp. Immunol. **32,** 654-663.

Handfield, M., Simard, P., Coullard, M. and Letarte, R. 1996. *Aeromonas hydrophila* isolated from food and drinking water: hemagglutination, hemolysis, and cytotoxicity for a human intestinal cell line (HT-29). Appl. Environ. Microbiol. **62**, 3459-3461.

Hansan, P.K., Lunestad, B.T. and Samuelsen, O.B. 1992. Ecological effects of antibiotics and chemotherapeutants from the fish farming. (Michel, C. and Alderman, D.J. Eds.). Off. Int. Epiz., Paris, 12–15.

Hansen, G.H. and Olafsen, J.A. 1989. Bacterial colonization of cod (*Gadus morhua* L.) and halibut (*Hippoglossus hippoglossus*) eggs in marine aquaculture. Appl. Environ. Microbiol. **55**, 1435- 1446

Hansen, J.D., Landis, E.D. and Phillips, R.B. 2005. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: implications for a distinctive B cell developmental pathway in teleost fish. Proc. Natl. Acad. Sci. U.S.A. **102**, 6919–6924.

Hanson, L.A. and Grizzle, J.M. 1985. Nitrite- induced predisposition of channel catfish to bacterial diseases. Prog. Fish Cultur. **47**, 98-101.

Hardie, L.J., Ellis, A.E. and Secombes, C.J. 1996. In vitro activation of rainbow trout macrophages stimulates inhibition of *Renibacterium salmoninarum* growth concomitant with augmented generation of respiratory burst products. Dis. Aquat. Org. **25**, 175-83.

Hardie, L.J., Fletcher, T.C. and Secombes, C.J. 1990. The effect of vitamin E on the immune response of Atlantic salmon (*Salmo salar*). Aquaculture **87**, 1-13.

Hardie, L.J., Fletcher, T.C. and Secombes, C.J., 1991. The effect of dietary vitamin C on the immune response of Atlantic salmon (*Salmo salar*). Aquaculture **95**, 201–214.

Hardie, L.J., Laing, K.J., Daniels, G.D., Grabowski, P.S., Cunningham, C. and Secombes, C.J. 1998. Isolation of the first piscine transforming growth factor-ß gene: analysis reveals tissue specific expression and a potential regulatory sequence in rainbow trout (*Oncorhynchus mykiss*). Cytokine **10**, 555-563.

Harikrishnan, R. and Balasundaram, C. 2005. Modern trends in *Aeromonas hydrophila* disease management with fish. Rev. Fish. Sci. **13**, 281-320.

Harikrishnan, R., Rani, M.R. and Balasundaram, C. 2003. Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. Aquaculture **221**, 41-50.

Hassett, D.J. and Cohen, M.S. 1989. Bacterial adoption to oxidative stress: implications of pathogenesis and interaction with phagocytic cells, Fed. Am. Soc. Exp. Biol. **3,** 1574-1581.

Hazen, T.C. 1979. Ecology of *Aeromonas hydrophila* in a South Carolina cooling reservoir. Microb. Ecol. **5,** 179-195.

Hazen, T.C., Raker, M.L., Esch, G.W. and Fliermans, C.B. 1982. Ultrastructure of re-sore lesions on largemouth bass (*Micropterus salmoides*); association of the ciliate *Epistylis* sp. and the bacterium *Aeromonas hydrophila*. J. Protozool. **25**, 351-355.

Henderson, R.J. 1996. Fatty acid metabolism in freshwater fish with particular reference to polyunsaturated fatty acids. Arch. Tierernahr. **49**, 5-22.

Hettiarachechi, D.C., and Cheong, C.H. 1994. Some characteristics of *Aeromonas hydrophila* and *Vibrio* species isolated from bacterial disease outbreaks in ornamental fish culture in Sri Lanka. J. Natl. Sci. Counc. Sri. Lanka. **22**: 361–369.

Hidalgo, M.C., Urea, E. and Sanz, A. 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. Aquaculture **170**, 267-283.

Hille, S. 1982. A literature review of the blood chemistry of rainbow trout, *Salmo gairdneri* rich. J. Fish. Biol. **20**, 535-569.

Hirvelae-Koshi, V., Koshi, P. and Nirranen, H. 1994. Biochemical properties and drug resistance of *Aeromonas salmonicida* in Finland. Dis. Aquat. Org. **20**, 191-196.

Hoar, W.S., Parrell, A.P. and Randall, D.J. 1992. The cardiovascular system. In; Fish physiology. Acadmic Press. London.Vol. 12. Pp. 85- 92.

Hofer, R. 1979. The adaptation of digestive enzymes to temperature, season and diet in roach, *Tutilus urtilus* L. and rudd *Scardinius erthropthalmus* L. 1. Amylase. J. Fish Biol. **14**, 565-572.

Holland, M.C.H. and Lambris, J.D. 2002. The complement system in teleosts. Fish Shellfish Immunol. **12**, 399-420.

Holmes, P., Niccols, L.M. and Sartory, D.P. 1996. The ecology of mesophilic *Aeromonas* in the aquatic environment. In the genus *Aeromonas*, ed. B. Austin, Altwegg. M., and Gosling P.J. Chichester: Wiley**.** pp. 127-50.

Hong, S., Zou, J., Collet, B., Bols, N.C. and Secombes C.J. 2004. Analysis and characterisation of IL-1ß processing in rainbow trout, *Oncorhynchus mykiss*. FishShellfish Immunol. **16,** 453-9.

Hosoi, T., Hirose, R., Saegusa, S., Ametani, A., Kiuchi, K. and Kaminogawa. S. 2003. Cytokine responses of human intestinal epithelial-like Caco-2 cells to the nonpathogenic bacterium *Bacillus subtilis* (natto). Int. J. Fd. Microbiol. **82**, 255-264.

Hossain, M.A., Focken, U. and Becker, K., 2001. Effect of soaking and soaking followed by autoclaving of Sesbania seeds on growth and feed utilization in common carp, *Cyprinus carpio* L. Aquaculture **203**, 133-148.

Houwen, B. 2002. Blood film preparation and staining procedures. Clin. Lab. Med. **22**, 1–14.

Howard, S.P. and Buckley, J.T. 1985. Activation of the hole-forming toxin aerolysin by extracellular processing. J. Bacteriol. **163**, 336- 340.

Hoyle, R.J. 1973. Digestive enzyme secretion after dietary variation in the American lobster (*Homarus americanus*). J. Fish. Res. Bd. Can. **30,** 1647-1653.

Hudec, J., Burdovaa, M., Kobida, L., Komora, L., Macho, V., Kogan, G., Turianica, I., Kochanvaa, R., Lozyek, O., Hebaa, M. and Chlebo, P. 2007. Antioxidant capacity changes and phenolic profile of *Echinacea purpurea*, nettle (*Urtica dioica* L.), and dandelion (*Taraxacum officinale*) after application of polyamine and phenolic biosynthesis regulators. J. Agric. Food Chem. **55**, 5689-5696.

Huizinga, H.W., Esch, G.W. and Hazen, T.C. 1979. Histopathology of red-sore disease (*Aeromonas hydrophila*) in naturally and experimentally infected largemouth bass *Micropterus salmoides* (Lacépède). J. Fish Dis. **2**, 263 - 277.

Ibrahem, M.D., Mostafa, M.M., Arab, R.M.H. and Rezk, M.A. 2008. Prevalence of *Aeromonas hydrophila* infection in wild and cultured Tilapia (*O. niloticus*) in Egypt. International Symposium on Tilapia in Aquaculture **8**, 1257-1271.

Inglis, V., Richards, R.H. and Woodwar, K.N. 1993. Motile *Aeromonad* septicaemia. In Bacterial Diseases of Fish. (eds. Inglis, V., Roberts, R.J. and Bromage, N.R.), pp. 143-152. Blackwell Scientific Publications, London.

Ingram, G.A. 1980. Substances involved in the natural resistance of fish to infection a review. J. Fish Biol. **16**, 23-60.

Irianto, A. and Austin, B. 2002. Probiotics in aquaculture, Review. J. Fish Dis. **25**, 633-642.

Ispir, U. 2009. Prophylactic effect of levamisole on rainbow trout (*Oncorhynchus mykiss*) against *Yersinia ruckeri*. Pesq. Vet. Bras. **29,** 700-702.

Itou, T., Lida, T. and Kawatsu, H. 1996. Kinetics of oxygen metabolism during respiratory burst in Japanese eel neutrophils. Dev. Comp. Immunol. **20**, 323-330.

Iwama, G. and Nakanishi, T. 1996. The fish Immune System; Organisms, Pathogen and Environment. Academic Press, London, UK.

Jamroz, D. 2005. Nutritional factors supporting the immune response in animal. Krmiva **42**, 207-219.

Jeney, G. and Anderson, D.P. 1993a. Glucan injection or bath exposure given alone or in combination with a bacterin enhance the non-specific defence mechanisms in rainbow trout (*Oncorhyncus mykiss*). Aquaculture.**116,** 315-329.

Jeney, G. and Anderson, D.P. 1993b. Enhanced immune response and protection in rainbow trout to *Aeromonas salmonicida* bacterin following prior immersion in stimulates. J. Fish. Shellfish. Immunol. **3**, 51-58.

Jenkins, J.A. and Ourth, D.D. 1993. Opsonic effect of the alternative complement pathway on channel catfish peripheral blood phagocytes. Vet. Immunol. Immunopathol. **39**, 447-459.

Jeorg, H.G. and lee, Y.W. 1998. Protective effect of daily sulfide on N-nitrosodimethylamine- induced immunosuppression in mice. Cancer Lett. **11,** 73-79.

Jha, A.K., Pal, A.K., Sahu, N.P., Kumara, S. and Mukherjeea, S.C. 2007. Haemato-immunological responses to dietary yest RNA, w-3 fatty acid and ß-carotene in *Catla catla* juveniles. Fish Shellfish Immunol. **23**, 917-927.

Ji, S., Jronh, G., IM, G., Lee, S., Yoo, J. and Takii, K. 2007. Dietary medicinal herbs improve growth performance, fatty acid utilization, and stress recovery of Japanese flounder. Fish. Sci. **73**, 70-76

Jia, L., He, X. and Yang, Y. 1991. Evaluation of partial replacement of fish meal and soybean meal cake by alfalfa, *Trifoliun* sp., in practical diets for Chinese blunt snout bream, *Megalobrama amblycephala* fingerlings. In: Proceedings of the 4th Fish Nutrition. Research Workshop in Asia. (Ed.) De-Silva, S.S., No.5. pp. 119-123.

Jian, J. and Wu, Z. 2004. Influence of traditional Chinese medicine on non specific immunity of Jian Carp (*Cyprinus carpio* var. Jian). Fish Shellfish Immunol. **16**,185-191.

Jian, J. and Wu, Z. 2003. Effects of traditional Chinese medicine on nonspecific immunity and disease resistance of large yellow croaker, *Pseudosciaena crocea* (Richardson). Aquaculture **218**, 1-9.

Jiang, H., Siegel, J.N. and Gewurz, H. 1991. Binding and complement activation by C-reactive protein via the collagen-like region of C1q and inhibition of these reactions by monoclonal antibodies to C-reactive protein and C1q. J. Immunol. **146**, 2324-2330.

Jiravanichpaisal, P., Lee, S.Y., Kim, Y., Andrena, T. and Soderhall, I. 2007. Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: Characterization and expression pattern. Devel. Comp. Immunol. **31,** 441-455

Johnston, R.B. 1978. Oxygen metabolism and the microbicidal activity of macrophages. Fed. Proc. **37**, 2759-2764.

Johnsona, A.K., Harmsb, C.A., Levinea, J.F. and McHugh, J. 2006. A quantitative real-time RT-PCR assay to measure TGF-ß mRNA and its correlation with hematologic, plasma chemistry and organo-somatic indices responses in triamcinolone-treated Atlantic menhaden, *Brevoortia tyrannus*. Devel. Comp. Immunol. **30,** 473-484

Jolles, P. and Jolles, J. 1984. What's new in lysozyme research? Always a model system, today as yesterday. Mol. Cell Biochem. **63**, 165-89.

Joseph, J.K. and Abolaji, J. 1997. Effects of replacing maize with graded levels of cooked Nigerian mango seed kernels (*Mangifera indica*) on the performance, carcass yield and meat quality of broiler chickens. Biores. Technol. **61**; 99-102.

Juan, H., Tang, R., Wu, T. and Yu, K. 2000. Isolation of *Aeromonas hydrophila* in children with diarrhea. J. Microbiol. Immunol. Infect. **33**, 115- 117.

Kabuki, T., Nakajima, H., Arai, M., Ueda, S., Kuwabara, Y. and Dosako, S. 2000. Characterization of novel antimicrobial compounds from mango (*Mangifera indica* L.) kernel seeds. Fd. Chem. **71**, 61-66.

Kajita, Y., Sakai, M., Atsuta, S. and Kobayashi, M. 1990. The immunostimulatory effects of levamisole on rainbow trout, *Oncorhynchus mykiss*. Fish Pathol. **25**, 93-98.

Kajita, Y., Sakai, M., Atsuta, S. and Kobayashi, M., 1992. Immunopotentiantion activity of Freund's complete adjuvant in rainbow trout *Oncorhynchus mykiss*. Nipp. Suis. Gakk. **58**, 433-437.

Kapetanović, D. and Teskeredžić, E. 2004. *Aeromonas* spp bacteremia of rainbow trout fry (*Oncorhynchus mykiss*): biochemical characterization of the causative organism and antimicrobial susceptibility. Ribarstvo **62,** 95-102.

Kasper, D.L. and Barlam, T.F. 2005. Infections due to hacek group and miscellaneous gram negative bacteria. In: Kasper DL, Braunwald E, Fauci AS, *et al,* eds. Harrison's Principles of Internal Medicine. 16th ed . New York: McGraw-Hill. 862-870.

Katae, H., Kuono, K., Takase, Y., Miyazaki, H., Hashimoto, M. and Shimizu, M. 1979. The evaluation of piromidic acid as an antibiotic in fish: An in vitro and in vivo study. J. Fish Dis. **2,** 321- 335.

Kawahara, E., Sakai, M., Nomura, S., Chang, K.L. and Muraki, K. 1994. Immunomodulatory effects on white-spotted char, *Salvelinus leucomaenis*, injected with *Achromobacter stenohalis*. In: Chou, L.M. et al. (Eds.), The Third Asian Fisheries Forum. Asian Fisheries Society, Manila, Philippines, pp. 390–393.

Kawai, S. and Ikeda, S. 1973. Studies in digestive enzymes of fishes. III. Development of the digestive enzymes of rainbow trout after hatching and the effect of dietary change on the activities of digestive enzymes in the juvenile stage. Bull. Jpn. Soc. Sci. Fish. **38**, 265-270.

Kawakami, H., Shinohara, N. and Sakai, M. 1998. The non-specific immunostimulation and adjuvant effects of *Vibrio anguillarum* bacterin, M-glucan, chitin or Freund's complete adjuvant in yellowtail *Seriola quinqueradiata* to *Pasteurella piscicida* infection. Fish Pathol. **33**, 287–292.

Kennedy. S.B., Tucker, J.W., Neidic, C.L., Vermeer, G.K., Cooper, V.R., Jarrell, J.L. and Sennett, D.G. 1998. Bacterial management strategies for stock enhancement of warmwater marine fish: A case study with common snook (*Centropomus undecimalis*). Bull. Mar. Sci. **62**, 573-588.

Kiessling, K.H. and Kiessling, A. 1993. Selective utilization of fatty acids in rainbow trout (*Onchorhychus mykiss* Walbaum) red muscle mitochondria. Can. J. Zool. **71,** 248-251.

Kim, D. and Austin, B. 2006. Cytokine expression in leucocytes and gut cells of rainbow trout, *Oncorhynchus mykiss* Walbaum, induced by probiotics. Vet. Immunol. Immunopathol. **114**, 297–304.

Kim, K.H., Hwang, Y.J. and Bai, S.C. 1999. Resistance to *Vibrio alginolyticus* in juvenile rockfish (*Sebastes schlegeli*) fed diets containing different doses of aloe. Aquaculture **180**, 13- 21.

Kitao, T. and Yoshida, T. 1986. Effect of an immunopotentiator on *Aeromonas salmonicida* infection in rainbow trout (*Salmo gairdneri*). Vet. Immunol. Immunopathol. 12, 287-291.

Klaenhammer, T.R. 1988. Bacteriocins of lactic acid bacteria. Biochemistry **70**, 337-349.

Klebanoff, S.J. and Clark, R.D. 1978. *The Neutrophil: Function and Clinical Disorders*. Amsterdam: North Holland.

Kobayashi, K., Tomonaga, S. and Tanaka, S. 1992. Identification of a second immunoglobulin in the most primitive shark, the frill shark, *Chlamydoselachus anguineus*. Dev. Comp. Immunol. **16**, 295-299.

Koch, E. 2001. Extracts from fruits of saw palmetto (*Sabal serrulata*) and roots of stinging nettle (*Urtica dioica*): viable alternatives in the medical treatment of benign prostatic hyperplasia and associated lower urinary tracts symptoms. Planta Med. **67**, 489-500.

Kodama, H., Hirota, Y., Mukamoto, N., Baba, T. and Azuma, I., 1993. Activation of rainbow trout (*Oncorhynchus mykiss*) phagocytes by muramyl dipeptide. Dev. Comp. Immunol. **17**, 129-140.

Kodama, H., Matsuoka, Y., Tanaka, Y., Liu, Y., Iwasaki, T. and Watarai, S. 2004. Changes of C-reactive protein levels in rainbow trout (*Oncorhynchus mykiss*) sera after exposure to anti-ectoparasitic chemicals used in aquaculture. Fish Shellfish Immunol. **16,** 589-597.

Kodama, H., Yamada, F., Murai, T., Nakanishi, Y., Mikami, T. and Izawa, H. 1989. Activation of trout macrophages and production of CRP after immunization with *Vibrio anguillarum*. Dev. Comp. Immunol. **13**, 123–132.

Komatsu, K., Tsutsui, S., Hino, K., Araki, K., Hiura Y., Yamamoto, A., Nakamura, O. and Watanabe, T. 2009. Expression profiles of cytokines released in intestinal epithelial cells of the rainbow trout, *Oncorhynchus mykiss*, in response to bacterial infection. Dev. Comp. Immunol. **33**, 499-506.

Krogdahl, Å., Hemre, G.I. and Mommsen, T.P. 2005. Carbohydrates in fish nutrition: digestion and absorption in post larval stages. Aquacult. Nutr. **11,** 103-122.

Kumari, J. and Sahoo, P.K. 2005. Effects of cyclophosphamide on the immune system and disease resistance of Asian catfish *Clarias batrachus.* Fish Shellfish Immunol. **19**, 307-316.

Kusuda, R. and Salati, F. 1993. Major bacterial diseases affecting mariculture in Japan. Annu. Rev. Fish Dis. **3**, 69-85.

Kuz'mina, V.V. and Smirnova, Y.G. 1992. Distribution of alkaline phosphatase activity along the length of the intestine of freshwater teleosts. J. Ichthyol. **32,** 1-9.

Kuz'mina, V.V. 1990. Temperature influence on the total level of proteolytic activity in the digestive tract of some species of freshwater fishes. J. Ichthyol. **30,** 97-109.

Laing, K.J., Cunningham, C. and Secombes, C.J. 2000. Genes for three different isoforms of transforming growth factor-ß are present in plaice (*Pleuronectes platessa*) DNA. Fish Shellfish Immunol. **10**, 261-271.

Laing, K.J., Zou, J., Wang, T., Bols, B., Hirono, I., Aoki, T. and Secombes, C.J. 2002. Identification and analysis of an interleukin 8- like molecule in rainbow trout (*Oncorhynchus mykiss*). Dev. Comp. Immunol. **26**, 433-444.

Lall, S.P. 2002. The minerals. In: Fish Nutrition. (3rd Edn.), Halver, J.E. and Hardy, R.W. Academic Press, London. pp. 259-301.

Lall, S.P. and Olivier, G. 1993. Role of micronutrients in immune response and disease resistance in fish. In: Fish Nutrition in Practice (Kaushik, S.J., Luquet, P., Eds.), pp.101-118. INRA, Paris.

Lallier, R., Leblanc, D., Mittal, K.R. and Olivier, G. 1981. Serogrouping of motile *Aeromonas* species isolated from healthy and moribund fish. J. Appl. Environ. Microbiol. **42**, 56 - 60.

Lamas, J. and Ellis, A.E. 1994. Atlantic salmon (*Salmo salar*) neutrophil responses to *Aeromonas salmonicida*. Fish Shellfish Immunol. **4**, 201-219.

Lampart-Szczapa , E., Siger, A., Trojanowska, K., Nogala-Kalucka, M., Malecka, M. and Pacholek, B. 2003. Chemical composition and antibacterial activities of lupin seeds extracts. Mol. Nutri. Fd. Res. **47,** 286 – 290

Lange, S., Bjarnheidur, K., Gudmundsdottir, B. and Mangadottir, B. 2001. Humoral immune parameters of cultured Atlantic halibut (*Hippoglossus hippoglossus* L.). Fish Shellfish Immunol. **11**, 523-535.

Langston, A.L., Bricknell, I.R. and Ellis, A.E. 1998. Iron binding capacity of peripheral blood leucocyte lysates from Atlantic salmon (*Salmo salar* L.). In: Barnes A.C., Davidson G.A., Hiney M.P., McIntosh D., editors. Methodology in fish diseases research. Aberdeen: Fisheries Research Services; p. 111- 116.

Larsen, J.L. and Jensen, N.J. 1977. An *Aeromonas* species implicated in ulcer disease of the cod (*Gadus morhua*). Nord. Veterinaermed. **29**, 199-211.

Larsen, A.N., Solstad, T., Svineng, G., Seppola, M. and Jørgensen.,T. 2009. Molecular characterisation of a goose-type lysozyme gene in Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol. **26,** 122-132.

Larsen, M.H., Larsen, J.L. and Olren, J.E. 2001. Chemotaxin of *Vibro anguillarum* of fish mucus, role of the origin of the fish species and the serogroup of the pathogen. Microb. Ecol. **38**, 77-80.

Laskowski, M. and Kato, I. 1980. Protein inhibitors of proteinases. Ann. Rev. Biochem. **49,** 593- 626.

Lawrence, D.A. 1996. Transforming growth factor-beta: a general review. Eur. Cytokine Netw. **7,** 363-374.

Le Breton, A.D. 2009. Vaccines in Mediterranean aquaculture: Practice and needs. Options Méditerranéennes. **86,** 147- 154.

Leano, E.M., Inglis, V.B.M. and MacRac, I.H. 1999. Antibiotics resistance of *Vibrio* spp. and *Aeromonas* spp. isolated from fish and shrimp tissues and rearing water in Panay Island, Philippines. UPV J. Natl. Sci. **3,** 25-28.

Lee, K.J., Dabrowski, K., Blom, J.H., Bai, S.C. and Stromberg, P.C. 2002. A mixture of cottonseed meal, soybean meal and animal byproduct mixture as a fish meal substitute: Growth and tissue gossypol enantiomer in juvenile rainbow trout (*Onorhynchus mykiss*). J. Anim. Physiol. Anim. Nutr. **86**, 201-213.

Leiro, J., Ortega, M., Estevez, J., Ubeira, F.M. and Sanmartin, M.L. 1996. The role of opsonization by antibody and complement in vitro phagocytosis of microsporidian parasites by turbot spleen cells. Vet. Immunol. Immunopathol. **51,** 201-210.

Lemaitre, C., Orange, N., Saglio, P., Saint, N., Gagnon, I. and Molle, G. 1996. Characterisation and ion channel activities of novel antibacterial proteins from the skin mucosa of carp (*Cyprinus carpio*). Eur. J. Biochem. **240**, 143-149.

Lemieux, H., Blier, P. and Dutil, J.D. 1999. Do digestive enzymes set a physiological limit on growth rate and food conversion efficiency in the Atlantic cod (*Gadus morhua*)? Fish Physiol. Biochem. **20**, 293- 303.

Letterio, J.J. and Roberts, A.B. 1998. Regulation of immune responses by TGF-beta. Annu. Rev. Immunol. **16**, 137-161.

Lewis, L.P. and Galtin, D.M., 2004. Dietary oligonucleotides from yeast RNA influence immune responses and resistance of hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) to *Streptococcus iniae* infection. Fish Shellfish Immunol. **16**, 561-569.

Lewus, C.B., Kaiser, A. and Montville, T.J. 1991. Inhibition of foot-borne bacteral pathogens by bacteriocins from lactic acid bacteria isolated from meat. Appl. Environ. Microbiol. **57,** 1683-1688.

Li, G., Guo, Y., Zhao, D., Qian, P., Sun, J., Xiao, C., Liang, L. and Wang, H. 2006. Effects of levamisole on the immune response and disease resistance of *Clarias fuscus*. Aquaculture **253**, 212-217.

Li, P., Lewis, D.H. and Galtin, D.M. 2004. Dietary oligonucleotides from yeast RNA influence immune responses and resistance of hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) to *Streptococcus iniae* infection. Fish Shellfish Immunol. **16,** 561-569.

Li, Y. and Lovell, R.T. 1985. Elevated levels of dietary ascorbic acid increase immune responses in channel catfish. J. Nutr. **115**, 123-131.

Lie, Ø., Evensen, Ø., Sørensen, A. and Frøysadal, E. 1989. Study on lysozyme activity in some fish species. Dis. Aquat. Org. **6,** 1-5.

Lillehaug, A. 1991. Vaccination of Atlantic salmon (*Salmo salar* L.) against cold water vibriosis-duration of protection and effect on growth rate. Aquaculture. **92,** 99-107.

Lilley, J.H., Hart, D., Richards, R.H., Roberts, R.J., Cerenius, L. and Soderhall, K. 1997. Pan-Asian spread of single fungal clone results in large-scale fish kills. Vet. Rec. **140**, 653-654.

Lindsay, G. 1986. The significance of chitionlytic enzyme and lysozyme in rainbow trout (*Salmo gairdneri*) defence. Aquaculture **51**, 169- 173.

Ling-Yang, C., and Chiu-Yung, C. 1987. *Aeromonas hydrophila* from a tail rot disease of cultured black porgy in Taiwan. Coun. Agri. Fish Ser. **10,** 65-69.

Llobrera, A. and Gacutan, R.Q. 1987. *Aeromonas hydrophila* associated with ulcerative disease epizootic in Laguna de Bay, Philippines. Aquaculture **67,** 273-278.

Lopez-Lopez, S., Nolasco, H., Villarreal-Colmenares, H. and Civera-Cerecedo, R. 2005. Digestive enzyme response to supplemental ingredients in practical diets for juvenile freshwater crayfish *Cherax quadricarinatus*. Aquacult. Nutri **11,** 79-85.

Low, C., Wadsworth, S., Burrells, C. and Secombes, C.J. 2003. Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet. Aquaculture **221,** 23-40.

Lundblad, G., Fange, R., Slettengren, K. and Lund, J. 1979. Lysozyme, chitinases and exo-N-acetyl β-D-glucosamindase (NAGase) in lymphomyeloid tissue of marine fish. Mar. Biol. **53**, 311-315.

Lunden, T. and Bylund, G. 2000. The influence of in vitro and in vivo exposur to antibiotics on mitogeninduced proliferation of lymphoid cells in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol. **10,** 395-404,

Lydyard, P., Whelan, A. and Fanger, M. 2004. Immunology , 2nd ed., Taylor and Francis Group.

MacCrimmon, H.R. 1971. World Distribution of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Bd. Can. **28,** 663-704.

Magnadóttir, B. 2006. Innate immunity of fish (overview). Fish Shellfish Immunol. **20**, 137-151.

Maisuthisakul, P. 2009. Antioxidant potential and phenolic constituents of mango seed kernel from various extraction methods. Kasetsart University Annual Conference, Bangkok, Thailand.

Makare, N., Bodhankar S. and Rangari, V. 2001. Immunomodulatory activity of alcoholic extract of *Mangifera indica* L. in mice. J. Ethnopharmacol. **78;** 133-137.

Makkar, H.P.S. and Becker, K. 1999. Plant toxins and detoxification methods to improve feed quality of tropical seeds – review. Asian- Aust. J. Anim. Sci. **12**, 467-480.

Maqsood, S., Samoon, M.H. and Singh, P. 2009. Immunomodulatory and growth promoting effect of dietary levamisole in *Cyprinus carpio* fingerlings against the challenge of *Aeromonas hydrophila*. Turk. J. Fisher. Aqua. Sci. **9**, 111-120.

Marchalonis, J.J. 1969. Isolation and characterization of immunoglobulin-like proteins of the Australian lungfish *Neoceratodus forsteri*. Aust. J. Exp. Biol. Med. Sci. **47,** 405-419.

Marchesia, M., Parolinia, C., Diania, E., Rigamontia, E., Cornellia L., Arnoldia, A., Cesare R.S. and Chiesa G. 2008. Hypolipidaemic and anti-atherosclerotic effects of lupin proteins in a rabbit model. Brit. J. Nutr. **100**, 707-710.

Martem'yanov, V.I. 2001. Ranges of regulation of sodium, potassium, calcium, magnesium concentrations in plasma, erythrocytes, and muscle tissue of *Rutilus rutilus* under natural conditions. J. Evol. Biochem. Physiol. **37**, 141-147.

Martem'yanov, V.I. and Zaprudnova, R.A. 1982. Dynamics of electrolyte concentration in the blood plasma, red blood cells, and muscle tissue of fresh-water fish during stress. Biol. Nauki. **10**, 44-49.

Martem'yanov, V.I. 1983. Dynamics of electrolyte C=concentrations in fresh-water fish during stress, *Presnovodnaya fauna i biologiya gidrobiontov* (Fresh-Water Fauna and Biology of Hydrobionts). Leningrad. pp. 237-248.

Martins, M.L., Moraes, F.R., Miyazaki, D.M., Brum, C.D., Onak, E.M., Fenerick J.J. and Bozzo F.R. 2002. Alternative treatment for *Anacanthorus penilabiatus* (*Monogenea*: Dactylogyridae) infection in cultivated pacu, *Piaractus mesopotamicus* (*Osteichthyes*: *Characidae*) in Brazil and its haematological effects. Parasite **9,** 175-180.

Matsuo, K. and Miyazono, I., 1993. The influence of long-term administration of peptidoglycan on disease resistance and growth of juvenile rainbow trout. Bull. Jap. Soc. Sci. Fish. **59**, 1377-1379.

Mead, A.R. 1969. *Aeromonas hydrophila* in the leukoderma syndrome of *Achatina fulica*. Malacologia **9,** 43- 48.

Medzhitov, R. and Janeway, C.A. 2002. Decoding the patterns of self and nonself by the innate immune system. Science **296**, 298-300.

Medzhitov, R, and Janeway, C.A. 1998. An ancient system of host defence. Curr. Opin. Immunol. **10**, 12-15.

Meyer, F.P. 1970. Seasonal fluctuations in the incidence of disease on fish farms. In S.F. Snieszko, ed. A symposium on diseases of fishes and shellfishes. pp; 21-29. American Fisheries Society Special Publication 5. Bethesda.

Midtlyng, P.J., Reitan, L.J. and Speiliberrg, L. 1996. Efficacy and side effects of injectable furunculosis vaccines in Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol. **6,** 335-350.

Mitchell, A.J. and Plumb, J.A. 1980. Toxicity and efficacy of furanace on channel catfish *Ictalurus punctatus* (Rafinesque) infected experimentally with *Aeromonas hydrophila*. J. Fish Dis. **3,** 93 - 99.

Mohamad, S. and Abasali, H. 2010. Effect of plant extracts supplemented diets on immunity and resistance to *Aeromonas hydrophila* in common carp (*Cyprinus carpio*). Agric. J. **5,** 119-127

Morales, A.E., Cardenete, G., de la Higuera, M. and Sanz, A. 1994. Effects of dietary protein source on growth, feed conversion and energy utilization in rainbow trout (*Oncorhynchus mykiss*). Aquaculture **124**, 117-126.

Moriarty D.J.W. 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture **164**, 351-358.

Moriarty, D.J. 1973. The physiology of digestion of bluegreen algae in the cichlid fish, *Tilapia nilotica*. J. Zool. Lond. **171,** 25-39

Mukaida, N., Harada, A. and Matsushima, K., 1998. Interleukin-8 (IL-8) and monocytes chemotactic and activating factor (MCAF/MCP- 1), chemokines essentially involved in inflammatory and immune reactions. Cytokine Growth Factor Rev. **9,** 9–23.

Mulero, V., Estaban, M. A. and Meseguer, J. 1998. In vitro levamisole fails to increase sea bream (*Sparus aurata* L.) phagocyte functions. Fish Shellfish Immunol. **8**, 315-318.

Munkittrick, K.R., and Leatherland, J.F. 1983. Haematocrit values in feral goldfish, *Carassius auratus* L. as indicators of the health of the population. J. Fish Biol. **23,** 153-162.

Munoz, M., Cedeno, R., Rodriguez, J., Van der Knapp, W.P.W., Mialhe, E. and Bachere, E. 2000. Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, *Penaeus vannamei*. Aquaculture **191**, 89-107.

Murai, T., Kodama, H., Nakai, M., Mikami, T. and Izawa, H. 1990. Isolation and characterisation of rainbow trout C-reactive protein. Dev. Comp. Immunol.**14,** 49-58.

Murray, C.K. and Fletcher, T.C. 1976. The immunohistochemical localisation of lysozyme in plaice (*Pleuronectes platessa* L.) tissues. J. Fish Biol. **9,** 329-334.

Nagase, G. 1964. Contribution to physiology of digestion in *Tilapia mossambica* Peters: digestive enzymes, and the effects of diets on their activity. Z. Vergl. Physiol. **49**, 270-284.

Nakanishi, Y., Kodama, H., Murai, T., Mikami, T. and Izawa, H. 1991. Activation of rainbow trout complement by C-reactive protein. Am. J.Vet. Res. **52**, 397-401.

Narvaez, E., Berendsen, J., Guzman, F., Gallardo, J.A. and Mercado, L. 2010. An immunological method for quantifying antibacterial activity in Salmo salar (Linnaeus, 1758) skin mucus.. Fish Shellfish Immunol. **28,** 235-239

Natalia, Y., Hashim, R., Ali , A. and Chong, A. 2004. Characterization of digestive enzymes in a carnivorous ornamental fish, the Asian bony tongue *Scleropages formosus* (Osteoglossidae). Aquaculture **233**, 305-320.

Nauta, A.J., Daha, M., van Kooten, C., and Roos, A. 2003. Recognition and clearance of apoptotic cells: a role for complement and pentraxins. Trends. Immunol. **24,** 148-154.

Navarre, O. and Halver, J.E. 1989. Disease resistance and humoral antibody production in rainbow trout fed high levels of vitamin C. Aquaculture **79**, 207-221.

Nayak, S.K., Swain, P., Nanda, P.K., Dash, S., Shukla, S. and Meher, P.K. 2008. Effect of endotoxin on the immunity of Indian major carp, *Labeo rohita*. Fish Shellfish Immunol. **24**, 394-399.

Ndong, D. and Fall, J. 2007. The effect of garlic (*Allium sativum*) on growth and immune responses of hybrid tilapia (*Oreochromis niloticus* x *Oreochromis aureus*), Document Scientifique du CRODT, p 1–22.

Newman, S.G. 1983. *Aeromonas hydrophila*: A review with emphasis of its role in fish diseases. In Antigens of Fish Pathogens: Development and Production for Vaccines and Serodiagnostics, ed. D.P. Anderson, M. Dorson and Ph. Dubouret, pp. 87-117. Lyon: Foundation Marcel Merieux.

Neves, J., Wilson, J.M. and Rodrigues, P.S. 2009. Transferrin and ferritin response to bacterial infection: The role of the liver and brain in fish. Devel. Comp. Immunol. **33,** 848-857.

Nielsen, B., Dalsgaard, I., Brown, D.J. and Larsen, J.L. 1994. *Aeromonas salmonicida* subsp. *salmonicida*: Correlation of protein patterns, antibiotics resistance, exoprotease activities, haemolysis and pathological lesions produced in vitro. J. Fish Dis. **17,** 387-397.

Nikl, L., Albright, L.J. and Evelyn, T.P.T., 1991. Influence of seven immunostimulants on the immune responses of coho salmon to *Aeromonas salmonicida*. Dis. Aquat. Org. **12,** 7-12.

Nikl, L., Evelyn, T.P.T. and Albright, L.J. 1993. Trials with an orally and immersion-administrated ß-1, 3 glucan as an immunoprophylactic against *Aeromonas salmonicida* in juvenile chinook salmon *Oncorhynchus tshawytscha*. Dis. Aquat. Org. **17,** 191-196.

Nonaka, M. 1994. Molecular analysis of the lamprey complement system. Fish Shellfish Immunol. **4,** 437-446.

Norqvist, A., Hagstrom, A. and Wolf-Watz, H., 1989. Protection of rainbow trout against vibriosis and furunculosis by the use of attenuated strains of *Vibrio anguillarum*. Appl. Environ. Microbiol. **55,** 1400-1405.

Nya, E.J. and Austin, B. 2009a. Use of garlic, *Allium sativum*, to control Aeromonas hydrophila infections in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. **32,** 963-970.

Nya, E. and Austin, B. 2009b. Use of dietary ginger, *Zingiber officinale* Roscoe, as an immunostimulant to control *Aeromonas hydrophila* infections in rainbow trout, *Oncorhynchus mykiss* (Walbaum) J. Fish Dis. **32**, 971-977.

Nya, E.J. and Austin, B. 2010. Use of bacterial lipopolysaccharide (LPS) as an immunostimulant for the control of *Aeromonas hydrophila* infections in rainbow trout *Oncorhynchus mykiss* (Walbaum). J. Appl. Microbiol. **108,** 686-694.

Okai, D.B. and Aboagye, J. 1990. The effects of mango seed kernel meal (MSKL) on the performance of growing rats. Biol. Wastes **34**, 171-175.

Olivier, G., Evelyn, T.P.T. and Lallier, R., 1985. Immunity to *Aeromonas salmonicida* in coho salmon (*Oncorhynchus kisutch*) induced by modified Freund's complete adjuvant: its non-specific nature and the probable role of macrophages in the phenomenon. Dev. Comp. Immunol. **9,** 419-432.

Olli, J., Hjelmeland, K. and Krogdahl, A. 1994. Soybean trypsin inhibitor in diets for Atlantic salmon (*Salmo salar*, L): Effects on nutrient digestibilities and in pyloric caeca homogenate and intestinal content. Comp. Biochem. Physiol. **109,** 923-928.

Ollier, W.E.R. 2004. Cytokine genes and disease susceptibility. Cytokine **28**, 174-178.

Olsson, J.C., Westerdahl, A., Conway, P.L. and Kjelleberg, S. 1992. Intestinal colonization potential of turbot (*Scophthalmus maximus*) and dab (*Limanda limanda*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl. Environ. Microbiol. **58,** 551-556.

Olvera-Novoa, M.A., Campos, S.G., Sabido, M.G. and Martinez-Palacios, C.A. 1990. The use of alfalfa leaf protein concentrates as a protein source in diets for tilapia (*Oreochromis mosssambicus*). Aquaculture **90,** 291-302.

Olvera-Novoa, M.A., Martinez-palacios, C.A., Galvan, C.R. and Chavez, S.C., 1988. The use of seed of the leguminous plant *Sesbania grandiflora* as a partial replacement for fish meal in diets for tilapia (*Oreochromis mossambicus*). Aquaculture **71,** 51-60.

Omoregie, E., Ufodike, E.B.C. and Umaru, M.S. 1991. Growth and feed utilization of *Orechromis niloticus* fingerlings fed diets containing cassava peelings and mango seeds. Aquabyte **4**, 6-7.

Ortuno, J., Esteban, M.A. and Meseguer, J. 1999. Effect of high dietary intake of Vitamin C on nonspecific immune response of gilthead sea bream (*Sparus aurata* L.). Fish Shellfish Immunol. **9,** 429-443.

Ortuno, J., Cuesta, A., Esteban, M.A. and Meseguer, J. 2001. Effect of oral administration of high vitamin C and E dosages on the gilthead seabream (*Sparus aurata* L.) innate immune system. Vet. Immunol. Immunopathol. **79**, 167-180.

Osborne, J.A., Fensch, G.E. and Charba, F.F. (1989).The abundance of *Aeromonas hydrophila* L. at Lake Harney on the St. Johns River with respect to red sore disease in striped mullet (*Mugil cephalus* L.) Florida Scienstist **52**, 171- 176.

Overman, T.L., Kessier, J.F. and Seabolt, J.P. 1985. Comparison of API 20E, API Rapid E, and API Rapid NFT for identification of members of the Family Vibrionaceae. J. Clin. Microbiol. **22**, 778-781.

Owens, L. 2003. Diseases. In: Aquaculture- Farming Aquatic Animals and Plants (Lucas J.S. and P.C. Southgate, eds.), pp.47- 73. Blackwell Publishing, Oxford, UK.

Palumbo, S., Abeya, C. and Stelma, G. 1992. *Aeromonas hydrophila* group. In: Compendium of methods for the microbiological examination of food, (Vanderzant C. and Splittstoesser, F. Eds.). Washington: Asian Pacific American Heritage Association. pp. 497–515.

Paniagua, C., Rivero, O., Anguita, J. and Naharro, G. 1990. Pathogenicity factors and virulence for rainbow trout (*Salmo gairdneri*) or motile *Aeromonas* spp. isolated from a river. J. Clin. Microbiol. **28,** 350- 355.

Panigrahia, A., Kirona, V., Satoha, S., Hironob, I., Kobayashib, T., Sugitac, H., Puangkaewa, J. and Aoki, T. 2007. Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding. Dev. Comp. Immunol. **31,** 372-382.

Parker, R.B. 1974. Probiotics, the other half of the antibiotic story. Anim. Nutr. Health. **29,** 4-8.

Parry, R.M., Chandan, R.C. and Shahani, R.M. 1965. A rapid sensitive assay of muramidase. Proc. Soc. Exp. Biol. Med. **119,** 384–386.

Pasquale, V., Baloda, S.B., Dumontet, S. and Krovacek, K. 1994. An outbreak of *Aeromonas hydrophila* infection in turtiles (*Pseudemis scripta*). Appl. Environ. Microbial. **68**, 1678–1680.

Pavasovic, A., Anderson, A.J. Mather, P.B. and Richardson, N.A. 2007. Effect of a variety of animal, plant and single cell-based feed ingredients on diet digestibility and digestive enzyme activity in redclaw crayfish, *Cherax quadricarinatus*. Aquaculture **272,** 564-572.

Peddie, S., Zou, J. and Secombes, C.J. (2002). Biologically active IL-1ß derived peptide stimulates phagocytosis and bactericidal activity in rainbow trout *Oncorhynchus mykiss* (Walbaum) head kidney leucocytes in vitro. J. Fish Dis. **25,** 351-360.

Petterson, D.S. and Mackintosh, J.B. (1994). The chemical composition of lupin seed grown in Australia. In: Dracup, J. ed., Proceedings of the First Australian Lupin Technical Symposium, Perth, Western Australia, 17–21 October 1994. Department of Agriculture, WA, Australia, pp. 39–48.

Pfaffi, M.W., Horgan, G.W. and Dempfle, L. 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucl. Acids Res. **30**, 1- 10.

Pfeffer, E., Kinzinger, S. and Rodehutscord, M. 1995. Influence of the proportion of poultry slaughter by-products and of untreated or hydrothermically treated legume seeds in diets for rainbow trout, *Oncorhynchus mykiss* (Walbaum), on apparent digestibilities of their energy and organic compounds. Aquacult. Nutr. **1,** 111-117.

Pianetti, A., Falcioni, T., Bruscolini, F., Sabatini, L., Sisti, E. and Papa, S. 2005. Determination of the viability of *Aeromonas hydrophila* in different types of water by

flow cytometry, and comparison with classical methods, Appl. Environ. Microbiol. **71,** 7948-7954.

Pierce, L.R., Palti, Y., Silverstein, J.T., Barrows, F.T., Hallerman, E.M. and Parsons, J.E. 2008. Family growth response to fishmeal and plant-based diets shows genotype diet interaction in rainbow trout (*Oncorhynchus mykiss*). Aquaculture **278,** 37-42.

Pillay, T.V.R. and Kutty, M.N. 2005. Aquaculture principle and practices. 2nd edn, pp. 7-9.

Pleguezuelos, O., Zou, J., Cunningham, C. and Secombes, C.J. 2000. Cloning, sequencing and analysis of expression of a second IL-1ß gene in rainbow trout (*Oncorhynchus mykiss*). Immunogenetics **51,** 1002-1011.

Plouffe, D.A., Hanington, P.C., Walsh, J.G., Wilson, E.C. and Belosevic, M. 2006. Comparison of select innate immune mechanisms of fish and mammals. Xenotransplantation **12**, 266-277.

Popovic, N.T., Rakovac, R.C. and Perovic, I.S. 2007. Commercial phenotypic tests (API 20E) in diagnosis of fish bacteria: a review. Vet. Med. **52,** 49-53.

Poppe, T.T. and Breck, O. 1997. Pathology of Atlantic salmon, *Salmo salar* intraperitoneally immunized with oil-adjuvanted vaccine. A case report. Dis. Aquat. Org. **29**, 219-226.

Punitha, S.M.J., Babu, M.M., Sivaram, V., Shankar, V.S. and Dhas, S.A. 2008. Immunostimulating influence of herbal biomedicines on nonspecific immunity in grouper *Epinephelus tauvina* juvenile against *Vibrio harveyiinfection*. Aquacult. Int. **16,** 511-523.

Purohit, S.S., and Mathur, S.K. 1999. Drugs in biotechnology fundamentals and applications. India: Maximillan Publishers.

Quade, M.J. and Roth, J.A. 1997. A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Vet. Immunol. Immunopathol. **58,** 239-248.

Queiroz, J.F. and Boyd, C.E. 1998. Effects of a bacterial inoculum in channel catfish ponds. J. Wld. Aquacult. Soc. **29**, 67-73.

Raa, J. 1996. The use of immunostimulatory substances in fish and shellfish farming. Rev. Fish Sci. **4**, 1-60.

Raa, R., Rørstad, G., Engstad, R. and Robertsen, B. 1992. The use of immunostimulants to increase resistance of aquatic organisms to microbial infections. In: Shariff, M., Subasighe, R.P. and Arthur, J.R. (Eds.), Diseases in Asian Aquaculture Vol. 1. Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 39–50.

Rao, Y.V. and Chakrabarti, R. 2004. Enhanced anti-proteases in *Labeo rohita* fed with diet containing herbal ingredients. Ind. J. Clin. Biochem. **19,** 132-134.

Rao, Y.V. and Chakrabarti, R. 2005. Stimulation of immunity in Indian major carp *Catla catla* with herbal feed ingredients. Fish Shellfish Immunol. **18,** 327-334.

Rao, Y.V., Das, B.K., Jyotyrmayee, P. and Chakrabarti, R. 2006. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. Fish Shellfish Immunol. **20,** 263 – 273.

Rath, R.K. 2000. Freshwater aquaculture. 2nd ed. Jodhpur, India: Scientific Publishers review of selected literature. J. Fish. Biol. **4,** 593-604.

Refstie, S., Førde-Skjærvik, O., Rosenlund, G. and Rørvik, K.A. 2006. Feed intake, growth, and utilisation of macronutrients and amino acids by 1- and 2-year old Atlantic cod (*Gadus morhua*) fed standard or bioprocessed soybean meal. Aquaculture **255,** 279-291.

Richards, G.P. and Parveen, S. 2005. A survey for phosphoglucose isomerase with lysyl aminopeptidase activity in Vibrionaceae and non-*Vibrio* pathogens. Biochem. Biophys. A. **1748,** 128- 133.

Richter, N., Siddhuraju, P. and Becker, K. 2003. Evaluation of the quality of (*Moringa oleifera* Lam.) leaves as an alternative protein source for Nile tilapia (*Oreochromis niloliticus* L.). Aquaculture **217,** 599–611.

Roberts, R.J., Macintosh, D.J., Tonguthai, K., Boonyaratplain, S., Tayaputch, N., Phillips, M.J. and Nukkar, S.D. 1986. Field and Laboratory Investigations into Ulcerative Fish Diseases in the Asia-Pacific Region. Bangkok: F.A.O.

Robertsen, B., Ehgstad, R.E. and Jørgensen, J.B. 1994. ß-glucan as immunostimulants in fish. In: Stolen, J.S., Fletcher, T.C. (Eds.), Modulators of Fish Immune Responses Vol. 1, SOS Publications, Fair Haven, NJ, pp. 83–99.

Robertsen, B., Rørstad, G., Engstad, R. and Raa, J. 1990. Enhancement of non-specific disease resistance in Atlantic salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. J. Fish Dis. **13,** 391-400.

Robertson, P.A., O'Dowd, C., Burrells, C., Williams, P. and Austin, B. 2000. Use of *Carnobacterium* sp. as probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). Aquaculture **185**, 235-243.

Roch, P. 1999. Defense mechanisms and disease prevention in farmed marine invertebrate. Aquaculture. **172,** 125-145.

Rodriguez, A., Esteban M.A. and Meseguer, J. 2003. Phagocytosis and peroxide release by sea bream (*Sparus aurata* L.) leucocytes in response to yeast cells. Anat. Rec. **272**, 415-423.

Rombout, J.H. and Joosten, E.P. 1998. Mucosal immunity. In: Pastoret P., Griebel P., Bazin H., Govaerts A., editors. Handbook of Vertebrate Immunology. New York: Academic Press. p. 39-40.

Rombout, J.H., Taverne, N., Van de Kamp, M. and Taverne-Thiele, A.J. 1993. Differences in mucus and serum immunoglobulin of carp (*Cyprinus carpio* L.). Dev. Comp. Immunol. **17**, 309-317.

Ronneseth, A., Wergeland, H.I. and Pettersen, E.F. 2007. Neutrophils and B-cells in Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol. **23,** 493-503.

Rørstad, G., Aasjord, P.M. and Robertsen, B. 1993. Adjuvant effect of a yeast glucan in vaccines against furunculosis in Atlantic salmon (*Salmo salar* L). Fish Shellfish Immunol. **3,** 179-190.

Rozen, S. and Skaletsky, H.J. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz, S., Misener, S. (Eds.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp. 365–386.

Rungruangsak, K. and Utne, F. 1981. Effect of different acidified wet feeds on protease activates in the digestive tract and on growth rate of rainbow trout (*Salmo gairdneri* Richardson). Aquaculture **22**, 67-79.

Saavedra, M.J., Guedes-Novais, S., Alves, A., Rema, P., Tacao, M., Correia, A. and Martinez-Murcia, A. 2004. Resistance to beta-lactam antibiotics in *Aeromonas hydrophila* isolated from rainbow trout (*Oncorhynchus my*kiss). Int. Microbiol. **7,** 207-211.

Sabapathy, U. and Teo, L.H. (1993) A quantitative study of some digestive enzymes in rabbitfish, *Siganus canaliculatus* and the sea bass, *Lates calcarifer*. J Fish Biol 42, 595–602.

Sahu, S., Das, B.K., Mishra, B.K., Pradhan, J. and Sarangi, N. 2007a. Effect of *Magnifera indica* kernel as a feed additive on immunity and resistance to *Aeromonas hydrophila* in *Labeo rohita* fingerlings. Fish Shellfish Immunol. **23**, 109-118.

Sahu, S., Das, B.K., Mishra, B.K., Pradhan, J. and Sarangi, N. 2007b. Effect of *Allium* sativum on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. J. Appl. Ichthyol. **23,** 80-86.

Sairam, K., Hemalata, S., Kumar, A., Srinivasan, T., Ganesh, J. and Shankar, M. 2003. Evaluation of anti-diarrhoeal activity in seed extracts of *Mangifera indica*. J. Enthnopharmacol. **84**, 11-15.

Sakai, M. 1999. Current research status of fish immunostimulants. Aquaculture **172,** 63-92.

Sakai, M., Atsuta, S. and Kobayashi, M. 1995a. Efficacies of combined vaccine for *Vibrio anguillarum* and *Streptococcus* sp. Fish. Sci. **61,** 359-360.

Sakai, M., Kamiya, H., Atsuta, S. and Kobayashi, M. 1991. Immunodulatory effects on rainbow trout, *Oncorhynchus mykiss*, injected with the extract of abalone, *Haliotis discus* hannai. J. Appl. Ichthyol. **7,** 54-59.

Sakai, M., Kamiya, H., Ishii, S., Atsuta, S. and Kobayashi, M. 1992. The immunostimulating effects of chitin in rainbow trout, *Oncorhynchus mykiss*. In: Shariff, M., Subasighe, R.P., Arthur, J.R. (Eds)., Diseases in Asian Aquaculture Vol. 1. Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 413–417.

Sakai, M., Kobayashi, M. and Kawauchi, H., 1995b. Enhancement of chemiluminescent responses of Phagocytic cells from rainbow trout, *Oncorhynchus mykiss*, by injection of growth hormone. Fish Shellfish Immunol. **5,** 375-379.

Sakai, M., Kobayashi, M. and Yoshida, T., 1995d. Activation of rainbow trout, *Oncorhynchus mykiss*, phagocytic cells by administration of bovine lactoferrin. Comp. Biochem. Physiol. **110**, 755-759.

Sakai, M., Yoshida, T., Atsuta, S. and Kobayashi, M. 1995c. Enhancement of resistance to vibriosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum), by oral administration *Clostridium butyricum* bacterin. J. Fish Dis. **18,** 187-190.

Sakai, M., Yoshida, T. and Kobayashi, M. 1995e. Influence of the immunostimulant, EF203, on the immune responses of rainbow trout. *Oncorhynchus mykiss* to *Renibacterium salmoninarum*. Aquaculture. **138**, 61-67.

Salte, R., Gjoen, H.M., Norberg, K. and Gjedrem, T. 1993. Plasma protein levels as potential marker traits for resistance to furunculosis. J Fish Dis. 16, 561-568.

Samuel, M., Lam, T.J. and Sin, J.M. 1996. Effect of laminaran (β (1,3)-D-glucan) on the protective immunity of blue gourami, *Trichogaster trichopterus* against *Aeronmonas hydrophila*. J. Fish Shellfish Immunol. **6**, 443-454.

Sanarelli, G. 1891. On a new micro-organism of the water for which beasts pathogen with verandas and a constant temperature. Zbl. Bakt. Parasiten. **9,** 193-199 (in German).

Sandnes, K., Lie, O. and Waagbo, R. 1988. Normal ranges of some blood chemistry parameters in adult farmed Atlanic salmon, *Salmo salar*. J. Fish. Biol. **32**, 129-136.

Sano, T. 1998. Control of fish disease, and the use of drugs and vaccines in Japan. J. Appl. Ichthyol. **14,** 131-137

Santos, Y., Bandin, I. and Toranzo, A.E. 1996. Immunological analysis of extracellular products and cell surface components of motile *Aeromonas* isolated from fish. J. App. Bacteriol. **81,** 585-593.

Sanz, A., Morales, A.E., De la Higuera, M. and Cardenete, G. 1994. Sunflower meal compared with soybean meals as partial substitutes for fish meal in rainbow trout (*Oncorhynchus mykiss*) diets: protein and energy utilization. Aquaculture **128**, 287-300.

Sarder, M.R.I., Thompson, K.D., Penman, D.J. and McAndrew, B.J. 2001. Immune response of the Nile tilapia (*Oreochromis niloticus* L.) clones: 1. Non-specific responses. Dev. Comp. Immunol. **25,** 37-46.

Savan, R., Aman, A., Nakao, M., Watanuki, H. and Sakai, M. 2005. Discovery of a novel immunoglobulin heavy chain gene chimera from common carp (*Cyprinus carpio* L.). Immunogenetics. **57**, 458-463.

Scartezzini, P. and Speroni, E. 2002. Review on some plants of Indian traditional medicine with antioxidant activity. J. Ethnopharmacol. **71**, 23-43.

Schachte, J.H. 1978. Immunization of channel catfish, *Ictalurus punctatus*, against two bacterial diseases. Mar. Fish. Rev. **40,** 18 - 19.

Schmittou, H.R., Cremer, M.C. and Zhang, J. 2004. Principles and practices of high density fish culture in low volume cages. VI. Fish Stress, Health and Disease. pp. 1-11. American Soybean Association.

Schroder, M.B., Flano, E., Pilstrom, L. and Jorgensen, T.O. 1998. Localisation of Ig heavy chain mRNA positive cells in Atlantic cod (*Gadus morhua* L.) tissues; identified by in situ hybridisation. Fish Shellfish Immunol. **8,** 565-576.

Secombes, C.J. 1990. Isolation of salmonid macrophages and analysis of their killing activity. In: Techniques in Fish Immunology, Vol. 1 (ed. by J.S. Stolen, T.C. Fletcher, D.P. Anderson & B.S. Roberson), pp. 137–163. SOS Publications, Fair Haven, New Jersey.

Secombes, C.J., Bird, S. and Zou, J. 2005. Adaptive immunity in teleosts: cellular immunity. Dev. Biol. **121**, 25-32.

Secombes, C.J. 1996. The non-specific immune system: cellular defenses. In: Iwama G., Nakanishi T., editors. The fish immune system: organism, pathogen, and environments. CA: Academic press. pp. 63-103.

Secombes, C.J., Bird, S., Cunningham, C. and Zou, J. 1999. Interleukin-1 in fish. Fish Shellfish Immunol. **9,** 335-343.

Secombes, C.J., Wang, T., Hong, S., Peddie, S., Crampe, M., Laing, K.J., Cunningham, C. and Zou, J., 2001. Cytokines and innate immunity of fish. Dev. Comp. Immunol. **25,** 713-723.

Secombes, C.J., Zou, J., Hardie, L.J., Laing, K.J., Daniels, G.D. and Cunningham, C. 1997. Rainbow trout cytokine genes. Dev. Comp. Immunol. **21**, 138.

Selvaraj, V., Sampath, K. and Sekar, V. 2005. Administration of yeast glucan enhances survival and some non-specific and specific immune parameters in carp (*Cyprinus carpio*) infected with *Aeromonas hydrophila*. Fish Shellfish Immunol. **19,** 293-306.

Shalaby, A.M., Khattab, Y.A. and Abdel Rahman, A.M. 2006. Effects of garlic (*Allium sativum*) and chloramphenicol on growth performance, physiological parameters and survival of Nile tilapia (*Oreochromis niloticus*). J. Venom. Anim. Toxin Trop. Dis. **12,** 172-201.

Shao, J., Liu, J. and Xiang, L. 2004. *Aeromonas hydrophila* induces apoptosis in *Carassius auratus* lymphocytes in vitro. Aquaculture. **229,** 11-23.

Sharifuzzaman, S.M. and Austin, B. 2009. Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout. Fish Shellfish Immunol. **27,** 440-445.

Sharp, G.J. and Secombes, C.J. 1993. The role of reactive oxygen species in the killing of the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages. Fish Shellfish Immunol. **3,** 119-129.

Shieh, H.S. 1987. Protection of Atlantic salmon against motile aeromonad septicaemia with *Aeromonas hydrophila* protease. Microb. Lett. **36,** 133 - 138.

Shoemaker, C.A., Klesius, P.H. and Lim, C. 2001. Immunity and disease resistance in fish. In: Lim C., Webster CD. (Ed.)., Nutrition and Fish Health. Binghamton, New York; Food Products Press, pp. 149-162

Shotts, E.B. and Rimler, R. 1973. Medium for the isolation of *Aeromonas hydrophila*. Appl. Microbiol. **26,** 550-553.

Shotts, E.B., Gaines, J.L., Martin, L. and Prestwood, A.K. 1972. *Aeromonas* induced deaths among fish in a eutrophic inland lake. J. Am. Med. Assoc. **162,** 603-607.

Siddhuraju, P., Becker, K. and Makkar, H.P.S., 2000. Studies on the nutritional composition and antinutritional factors of three different germplasm seed materials of an under-utilised tropical legume, *Mucuna pruriens* var. utilis. J. Agric. Food Chem. **48,** 6048-6060.

Sigel, M.M., McCumber, L.J., Hightower, J.A., Hayasaka. S.S., Huggens, E.M. and Davis, J.F. 1983. *Ecteinascidia turbinata* extract activates components of inflammatory responses throughout the phylogenetic spectrum. Am. Zool. **23,** 221-227.

Sitjà-Bobadilla, A. Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S. and Pérez-Sánchez, J. 2005. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture **249,** 387-400.

Sivarama, V., Babua, M.M., Immanuela, G., Murugadassa, S., Citarasub, T. and Marian, M.P. 2004. Growth and immune response of juvenile greasy groupers (*Epinephelus tauvina*) fed with herbal antibacterial active principle supplemented diets against *Vibrio harveyi* infections. Aquaculture **237,** 9 -20.

Siwicki, A.K. 1987. Immunomodulating activity of levamisole in spawners carp (*Cyprinus carpio* L.). J. Fish Biol. **31,** 245-246.

Siwicki, A.K. 1989. Immunostimulating influence of levamisole on nonspecific immunity in carp (*Cyprinus carpio*). Dev. Comp. Immunol. **13,** 87-91.

Siwicki, A.K., Anderson, D.P. and Rumsey, G.L., 1994. Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. Vet. Immunol. Immunopathol. **41,** 125-139.

Skim, F., Lazrek, H.B., Kaaya, A., El-Amri, H. and Jana, M. 1999. Pharmacological studies of two antidiabetic plants: *Globularia alypum* and *Zygophyttum gaetulum*. Therapia **54,** 711-715.

Smith, V.J., Fernandes, J.M.O., Jones, S.J., Kemp, G.D. and Tatner, M.F. 2000. Antibacterial proteins in rainbow trout, *Oncorhynchus mykiss*. Fish Shellfish Immunol. **10,** 243-620.

Soivio, A. and Oikari, A. 1976, Haematological effects of stress on a teleost, *Esox lucius* L. J. Fish Biol. **8,** 397-411.

Soltan, M.A., Hanafy, M.A. and Wafa, M.I.A. 2008. Effect of replacing fish meal by a mixture of different plant protein sources in Nile tilapia (*Oreochromis niloticus* L.). Diets. Global Vet. **2,** 157-164.

Sorensen, K.K., Dahl, L.B. and Smedsrod, B. 1997. Role of endocardial endothelial cells in the turnover of hyaluronan in Atlantic cod (*Gadus morhua*). Cell Tissue Res. **290**, 101-109.

Spielmann, J., Shukla, A., Brandsch, C., Hirche, F., Stangl, G.I. and Eder. K. 2007. Dietary lupin protein lowers triglyceride concentrations in liver and plasma in rats by

reducing hepatic gene expression of sterol regulatory element-binding protein-1c. Ann. Nutr. Metab. **51,** 387-392.

Stafford, J.L. and Belosevic, M. 2003. Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation. Dev. Comp. Immunol. **27**, 539-554.

Stafford, J.L., Neumann, N.F. and Belosevic, M. 2001. Products of proteolytic cleavage of transferrin induce nitric oxide response of goldfish macrophages. Dev. Comp. Immunol. **25**,101-115.

Stenvik, J., Schroder, M.B., Olsen, K., Zapata, A. and Jorgensen, T.O. 2001. Expression of immunoglobulin heavy chain transcripts (VH-families, IgM and IgD) in head kidney and spleen of the Atlantic cod (*Gadus morhua* L.). Dev. Comp. Immunol. **25**, 291-302.

Stevenson, R.M.W. 1998. Vaccination against *Aeromonas hydrophila*. In: Fish vaccination, pp. 112–123 (A. E. Ellis, Ed.). London: Academic Press

Stickney, R.R., Hardy, R.W., Koch, K., Harrold, R., Seawright, D. and Massee, K.C. 1996. The effects of substituting selected oilseed protein concentrates for fish meal in rainbow trout (*Oncorhynchus mykiss*) diets. J. Wld. Aquacult. Soc. **27**, 57- 63.

Stoskopf, M.K. 1993. Fish medicine. Philadelphia, PA: Saunders W.B., pp. 473–474.

Subashkumar, R., Vivekanandhan, G., Raja, S.S., Natarajaseenivasan, K., Thayumanavan T. and Lakshmanaperumalsamy, P. 2007. Typing of *Aeromonas hydrophila* of fish and human diarrhoeal origin by outer membrane proteins and lipopolysaccharides. Ind. J. Microbiol. **47,** 46-50.

Subhadra, B., Lochmann, R., Rawles, S. and Chen, R. 2006. Effect of dietary lipid source on the growth, tissue composition and hematological parameters of largemouth bass (*Micropterus salmoides*). Aquaculture **255,** 210-222.

Sudaryono, A., Tsvetnenko, E., Hutabarat, J. and Evans, L.H. 1999. Lupin ingredients in shrimp (*Penaeus monodon*) diets: influence of lupin species and types of meals. Aquaculture **171**, 121-133.

Sugiura, S.H., Dong, F.M. and Hardy, R.W. 1998. Effects of dietary supplements on the availability of minerals in fish meal; preliminary observations. Aquaculture **160,** 283–303.

Sun, B.J., Wang, G.L., Xie, H.X., Gao, Q. and Nie P. 2006 Gene structure of goose-type lysozyme in the mandarin fish *Siniperca chuatsi* with analysis on the lytic activity of its recombinant in *Escherichia coli*. Aquaculture. 252,106-113

Suzer, C., Çoban, D., Okan, K.H., Saka, Ş., Firat, K., Otgucuoğlu, Ö. and Küçüksari, H. 2008. *Lactobacillus* spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.)

larvae: Effects on growth performance and digestive enzyme activities. Aquaculture **280**, 140-145.

Szalai, A.J., Bly, J.E. and Clem, L.W. 1994. Changes in serum concentration of channel catfish (*Ictalurus punctatus* Rafinesque) phosphorylcholine – reactive protein (PRP) in response to inflammatory agents, low temperature shock and infection by the fungus *Saprolegnia* sp. Fish Shellfish Immunol. **4,** 323-336.

Tanji, M., Kageyama, T. and Takahashi, K. 1988. Tuna pepsinogens and pepsins. Eur. J. Biochem. **177,** 251- 255.

Tengjaroenkul, B., Smith, B.J., Caceci, T. and Smith, S.A. 2000. Distribution of intestinal enzyme activities along the intestinal tract of cultured Nile tilapia, *Oreochromis niloticus* L. Aquaculture **182**, 317-327.

Tewary, A. and Patra, B.C. 2008. Use of vitamin C as an immunostimulant. Effect on growth, nutritional quality, and immune response of *Labeo rohita* (Ham.). Fish Physiol. Biochem. **34**, 251-259.

Thampuran, N., Surendran, P.K., Mukundan, M.K. and Gopakumar. K. 1995. Bacteriological studies on fish affected by epizootic ulcerative syndrome (EUS) in Kerala, India. Asian Fish. Sci. **8**, 103-111.

Thompson, I., Choubert, G., Houlihan, D.F. and Secombes, C.J. 1995. The effect of dietary vitamin A and astaxanthin on the immunocompetence of rainbow trout. Aquaculture **133,** 91-102.

Thompson, I., Fletcher, T.C., Houlihan, D.F. and Secombes, C.J. 1994. Effect of dietary vitamin A intake on the immunocompetence of Atlantic salmon (*Salmo solar* L). Fish Physiol. Biochem. **12,** 513-523.

Thompson, I., White, A. Fletcher, T.C., Houlihan D.F. and Secombes, C.J. 1993. The effect of stress on the immune response of Atlantic salmon (*Salmo salar* L.) fed diets containing different amounts of vitamin C, Aquaculture **114**, 1-18.

Thomson, A. 1994. The Cytokine Handbook, 2nd edition. Academic Press. London, pp 1-211.

Tian, J., Sun, B., Luo, Y., Zhang, Y. and Nie, P. 2009. Distribution of IgM, IgD and IgZ in mandarin fish, *Siniperca chuatsi* lymphoid tissues and their transcriptional changes after *Flavobacterium columnare* stimulation. Aquaculture. **288,**14-21

Toko, I.I., Fiogbe, E.D. and Kestemont, P. 2008. Mineral status of African catfish (*Clarias gariepinus*) fed diets containing graded levels of soybean or cottonseed meals. Aquaculture **275**, 298-305.

Treasure, J. 2003. Urtica semen reduces serum creatinine levels. J. Am. Herbalists Guild, **4,** 22-25.

Tulsidas, H., Ong, Y.Y. and Chan, K.C. 2008. *Aeromonas hydrophila* bacteraemia and portal pyaemia. Singapore Med. J. **49**, 346- 368.

Twibell, R.G. and Brown, P.B. 1998. Optimal dietary protein concentration for hybrid tilapia (*Oreochromis niloticus x Oreochromis aureus*) fed all plant diets. J. Wld. Aquacult. Soc. **29**, 9-16.

Twinning, S.S., Alexander, P.A., Huibregtse, K. and Glick, D.M., 1982. A pepsinogen from rainbow trout. Comp. Biochem. Physiol. **75**, 109-112.

Uys, W. and Hecht, T. 1987. Assays on the digestive enzymes of sharptooth catfish, *Clarias gariepinus* (Pisces: Clariidae). Aquaculture **63,** 303-313.

Vaddi, K., Keller, M. and Newton, R.C. 1997. The Chemokine Facts Book. Academic Press, London

Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstreate, W. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev. **64,** 655-671.

Vielma, J., Makinen, T., Ekholm, P. and Koskela, J. 2000. Influence of dietary soy and phytase levels on performance and body composition of large rainbow trout (*Oncorhynchus mykiss*) and algal availability of phosphorus load. Aquaculture **183,** 349-362.

Vine, N.G., Leukes, W.D. Kaiser, H., Daya, S., Baxter, J. and Hecht, T. 2004. Competition for attachment of aquaculture candidate probiotic and pathogenic bacteria on fish intestinal mucus. J. Fish Dis. **27,** 319-326.

Volpatti, D., D'Angelo, L., Jeney, G., Jeney, Z., Anderson, D.P. and Galeotti, M. 1998. Nonspecific immune response in fish fed glucan diets prior to induced transportation stress. J. Appl. Ichthyol. **14**, 201-206.

Von Gravenitz, A. and Mensch, A.H. 1968. The genus *Aeromonas* in human bacteriology. N. Engl. J. Med. **278,** 245 - 249.

Voss, E.W., Fryer, J.L. and Banowetz, G.M. 1978. Isolation, purification and partial characterisation of a lectin from chinook salmon ova. Arch. Bioch. Biophy. **186,** 25-34.

Wagner, H. and Proksch, A. 1985. Immunostimulatory Drugs of Fungi and Higher Plants in Economic and Medicinal Plant Research, volume 1, Academic Press - London - New York, pp. 113-153.

Wagner, H., Willer F. and Kreher, B. 1989. Biologically active compounds from the aqueous extract of *Urtica dioica*. Planta Med. **55,** 452-454.

Wahli, T., Verlhac, V. and Gabaudan, J. 1998. Influence of combined vitamins C and E on non-specific immunity and disease resistance of rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis. **21,** 127-137.

Wahli, T., Frischknecht, R., Schmitt, M., Gabaudan, J., Verlhac, V. and Meier, W. 1995. A comparison of the effect of silicone coated ascorbic acid and ascorbyl phosphate on the course of ichthyophthiriosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish Dis. **18,** 347-355.

Walter, H.E. 1984. Proteinases: methods with hemoglobin, casein and azocoll as substrates. In: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis, vol. V. Verlag Chemie, Weinheim, pp. 270- 277.

Wang, S.Y., Hsu, M.L., Hsu, H.C., Tzeng, C.H., Lee, S.S., Siao, M.S. and Ho, C.K. 1997. The anti-tumor effect of *Ganoderma lucidum* is mediated by cytokines released from activated macrophages and T lymphocytes. Int. J. Cancer. 70, 699-705.

Wang, T., Zou, J., Cunningham, C. and Secombes, C.J. 2002. Cloning and functional characterisation of the interleukin-1 beta 1 promoter of rainbow trout (*Oncorhynchus mykiss*). Biochim. Biophys. Acta. **1575**, 108-116.

Wang, G., Liu, Y., Li, F., Gao, H., Lei, Y. Liu, X. 2010. Immunostimulatory activities of *Bacillus simplex* DR-834 to carp (*Cyprinus carpio*). Fish Shellfish Immunol. (In press).

Warr, G.W. 1995. The immunoglobulin genes of fish. Dev. Comp. Immunol. **19**, 1-12.

Wassom, D.L. and Kelly, E.A. 1990. The role of the major histocompatibility complex in resistance to parasite infections. Crit. Rev. Immunol.**10**, 31-52.

Watanabe, T., Kiron, V. and Satoh, S. 1997. Trace minerals in fish nutrition. Aquaculture **151,** 185-207.

Watanabe, T. and Pongmaneerat, J. 1993. Potential of soybean meal as a protein source in extruded pellets for rainbow trout. Nipp. Suis. Gakk. **59,** 1415-1423.

Watanuki, H., Ota, K., Tassakka, A., Kato, T. and Sakai, M. 2006. Immunostimulant effects of dietary *Spirulina platensis* on carp, *Cyprinus carpio*. Aquaculture **258**, 157-163.

Wedemeyer, G.A. and Yasutake, W.T. 1977. Clinical methods for the assessment of the effects of environmental stress on fish health. Tech. Pap.U.S. Fish Wild. Sery. 89. In, Rand, G.M. and, Petrocelli, S.R., Fundametals of Aquatic Toxicology, Methods and Applications, Hemisphere, New York.

Weeks, B.A. and Warinner, J.E. 1986. Functional evaluation of macrophages in fish from a polluted estuary. Vet. Immunol. Immunopathol. **2,** 313-320.

Wells, R.M.G., Mclntyre, R.H., Morgan, A.K. and Davie, P.S. 1988. Physiological stress responses in big gamefish after capture; observations on plasma chemistry and blood factors. Comp. Biochem. Physicol. **84,** 565-571.

Wermenstam, N.E. and Pilstrom, L. 2001. T-cell antigen receptors in Atlantic cod (*Gadus morhua* L.): structure, organisation and expression of TCR alpha and beta genes. Dev. Comp. Immunol. **25**, 117-135.

Whittington, R.J., Munday, B.L., Akhlaghi, M., Reddacliff, G.L. and Carson, J. 1994. Humoral and peritoneal cell responses in rainbow trout (*Oncorhychus mykiss*) to ovalbumin, *Vibrio anguillarum* and Freund's complete adjuvant following intraperitoneal and bath immunisation. Fish Shellfish Immunol. **4,** 475-488.

Whyte, S.K. 2007. The innate immune response of finfish - a review of current knowledge. Fish Shellfish Immunol. **23**, 1127-1151.

Wiegertjes, G.F., Stet, R.J.M., Parmentier, H.K. and Van Muiswinkel, W.B. 1996. Immunogenetics of disease resistance in fish; a comparable approach, Dev. Comp. Immunol. **20**, 365-381.

Wilson, M.R. and Warr, G.W. 1992. Fish immunoglobulins and the genes that encode them. Ann. Rev. Fish Dis. **2,** 201-221.

Wong, C.Y.F., Heuzenroeder, M.W. and Flower, R.L. 1998. Inactivation of two haemolytic toxin genes in *Aeromonas hydrophila* attenuates virulence in a suckling mouse model. Microbiology **144**, 291- 298.

Wooley, R.E., Ritchie, B.W. and Burnley, V.V. 2004. In vitro effect of a buffered chelating agent and neomycin or oxytetracycline on bacteria associated with diseases of fish. Dis. Aquat. Org. **59,** 263-267 .

Worthington, V. 1993. Worthington Enzyme Manual. Enzymes and Related Biochemicals Worthington Chemical, New Jersey, US. 399 pp.

Wu, Y.Y., Rosati, R.R., Sessa, D. and Brown, P.B. 1994. Utilization of protein rich ethanol co-products from corn in tilapia feed. J. Am. Oil Chem. Soc. **71,** 1041-1043.

Xue, J., Xu, Y., Jin, L., Liu, G., Sun, Y., Li, S. and Zhang J. 2008. Effects of traditional Chinese medicine on immune responses in abalone, *Haliotis discus hannai* Ino. Fish Shellfish Immunol. **24,** 752-758.

Yambot, A.V. and Inglis, V. 1994. *Aeromonas hydrophila* isolated from Nile tilapia (*Oreochromis niloticus* L.) with "Eye Disease". International Symposium on Aquatic Animal Health, Seattle, WA (USA), 4 - 8 September 1994. University of California, School of Veterinary Medicine, Davis, CA. p.103.

Yano, T. 1992. Assay of hemolytic complement activity. In: Techniques in Fish Immunology (ed. by J.S. Stolen, T.C. Fletcher, D.P. Anderson, S.C. Hattari & A.F. Rowley), pp. 131–141. SOS Publications, Fair Haven, NJ.

Yano, T. 1996. The non-specific immune system: humoral defense. In: Iwama G, Nakanishi T, editors. The fish immune system: organism, pathogen, and environments. CA: Academic Press. pp. 106-157.

Yano, T., Mangindaan, R.E.P. and Matsuyama, H. 1989. Enhancement of the resistance of carp *Cyprinus carpio* to experimental *Edwardsiella tarda* infection, by some b-1, 3-glucans. Nipp. Suis. Gakk. **55**, 1815-1819.

Yin, G., Ardo, L., Thompson, K.D., Adams, A., Jeney, Z. and Jeney, G. 2009. Chinese herbs (*Astragalus radix* and *Ganoderma lucidum*) enhance immune response of carp, *Cyprinus carpio*, and protection against *Aeromonas hydrophila*. Fish Shellfish Immunol. **26**, 140-145.

Yin, G., Jeney, G., Racz, T., Xu, P., Jun, X. and Jeney, Z. 2006. Effect of two Chinese herbs (*Astragalus radix* and *Scutellaria radix*) on non specific immune response of tilapia, *Oreochromis niloticus*. Aquaculture **253**, 39-47.

Yoshida, T., Kruger, R. and Inglis, V. 1995. Augmentation of non-specific protection in African catfish, *Clarias gariepinus* (Burchell), by the long-term oral administration of immunostimulants. J. Fish Dis. **18,** 195-198.

Yousif, A.N., Albright, L.J. and Evelyn, T.P.T. 1994b. In vitro evidence for the antibacterial role of lysozyme in salmonid eggs. Dis. Aquat. Org. **19,** 15-19.

Yousif, A.N., Albright, L.J. and Evelyn, T.P.T. 1991. Occurrence of lysozyme in the eggs of coho salmon *Oncorhynchus kisutch*. Dis. Aquat. Org. **10,** 45-49.

Yousif, A.N., Albright, L.J. and Evelyn, T.P.T. 1994a. Purification and characterization of a galactose-specific lectin from the eggs of coho salmon *Oncorhynchus kisutch* and its interaction with fish pathogens. Dis. Aquay. Org. **20**, 127-136.

Yu, H.B., Rao, P.S.S., Lee, H.C., Vilches, S., Merino, S., Tomas, J.M. and Leung, K.Y. 2004. A Type III secretion system. Is required for *Aeromonas hydrophila* AH-1 pathogenesis. Infect. Immun. **72,** 1248–1256.

Yuan, C., Li, D., Chen, W., Sun, F., Wu, G., Gong, Y., Tang, J., Shen, M., and Han, X. 2007. Administration of a herbal immunoregulation mixture enhances some immune parameters in carp (*Cyprinus carpuio*). Fish Physiol. Biochem. **33,** 93- 101.

Yuan, C., Pan, X., Gong, Y., Xia, A., Wu, G., Tang, J., Han, X. 2008. Effects of *Astragalus* polysaccharides (APS) on the expression of immune response genes in head

kidney, gill and spleen of the common carp, *Cyprinus carpio* L. Int. Immunopharmacol. **8,** 51-58.

Yue, F., Pan, L., Miao, J., Zhang, L. and Li, J. 2010. Molecular cloning characterization and mRNA expression of two antibacterial peptides custin and anti-lipopolysaccharide factor in swimming crab *Portunus trituberculatus*. Comp. Biochem. Physiol. **156,** 77-85.

Zaki, M.S., Fawzi, O.M. and El-Jackey, J. 2008. Pathological and Biochemical Studies in *Tilapia nilotica* Infected with *Saprolegnia parasitica* and treated with Potassium Permanganate. American-Eurasian J. Agric. Environ. Sci. **3**, 677-680.

Zou, J., Peddie, S., Scapigliati, G., Zhang, Y., Bols, N.C. and Ellis, A.E. 2003. Functional characterisation of the recombinant tumor necrosis factor in rainbow trout (*Oncorhynchus mykiss*). Dev. Comp. Immunol. **27,** 813-822.

Zuo, X. and Woo, P.T.K. 1997. In vivo neutralization of *Cryptobia salmositica* metallo-protease by alpha 2-macroglobulin in the blood of rainbow trout *Oncorhynchus mykiss* and in brook charr *Salvelinus fontinalis*. Dis. Aquat. Org. **29**, 67-72.

Appendix

The formulae of the solutions and media used in this study are listed below. Unless otherwise indicated, the materials were stored at room temperature.

0.1 M KCl–HCl (pH 1.5)	A) 0.1 M solution of KCl (7.455 g in 1000 ml) B) 0.1 M HCl 50 ml of A + 33.3 ml of B diluted to a total of 200 ml
0.2 M glycine–HCl (pH 3.0)	A) 0.2 M solution of glycine (15.01 g in 1000 ml) B) 0.2 M HCl 50 ml of A + 11.4 ml of B diluted to a total of 200 ml
0.1MCitrate–0.2M phosphate (pH 7.0)	A) 0.1 M solution of citric acid (19.21g in 1000 ml) B) 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2 HPo_4 7H_2O$ **or** 71.7 g of $Na_2 HPO_4.12H_2O$ in 1000ml) 6.5 ml of A + 43.6 ml of B diluted to a total of 100
0.1 M glycine–NaOH (pH.10.0)	A) 0.1 M solution of glycine (7.505 g in 1000 ml) B) 0.1 M NaOH 50 ml of A + 32 ml of B diluted to a total of 200 ml
GPT reagent	2-Oxoglutrate (13 mmol/l) L-Alanine (440 mmol/lL) NADH (> 0.12 mmol/l) LDH (microbial) (> 2000 U/l)

	Tris buffer (97 mmol/l)
	EDTA (5.0 mmol/l)
GOT reagent	
Reagent 1	NADH (0.42 mmol/l)
	LDH (microbial) (> 150 U/l)
	2-Oxoglutrate (15 mmol/l)
	Tris buffer (47.5 mmol/l)
Reagent 2	L-Aspartate (1000 mmol/l)
	MDH (microbial) (> 500 U/l)
	Tris buffer (250.5 mmol/l)
Glucose reagent	Hexokinase (1000 U/l)
	G_6PDH (1000 U/l)
	ATP (1.0mM)
	NAD (1.0mM)
	Buffer pH 7.5 ± 0.1
50x TAE	Tris base =242 g
	Glacial acetic acid = 57.1 ml
	0.5M EDTA (pH: 8.0) --------- 100 ml
	Put the volume to 1 l
6x gel- loading buffer	40 % (w/v) sucrose
	0.25% (w/v) bromophenol blue
	0.25% (w/v) xylene cyanol FF; dissolved in distilled water. Stored at 4°C

10x MOPS	200 mM MOPS pH 7
	50 mM Na acetate
	10 mm EDTA
	Put the volume to 1l
Loading buffer for RNA	250 µl formamide
	83 µl formaldehyde
	50 µl 10x MOPS
	5 µl Ethidium bromide
	0.01% BPB (bromophenol blue)